1-10-2011

mGluR5 Involvement in Methamphetamine Reward and the Co-Morbidity of Schizophrenia and Stimulant Use Disorders

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mGluR5 INVOLVEMENT IN METHAMPHETAMINE REWARD AND THE CO-MORBIDITY OF SCHIZOPHRENIA AND STIMULANT USE DISORDERS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY

AMY ANNE HERROLD

CHICAGO, IL

DECEMBER 2010

ACKNOWLEDGEMENTS

I would like to extend my gratitude towards everyone who has helped and supported me in the process of my graduate school training. I would like to thank my advisor, Dr. T. Celeste Napier who has provided excellent guidance, support and training. She has helped me to seize many opportunities and pushed me to move my career in the right direction. I would also like to thank my committee, Drs. Edward J. Neafsey, Erika Piedras-Renteria, Anthony Grace and Michela Marinelli for their invaluable time, scientific advice and guidance. It has been a pleasure and an honor to have such a distinguished committee. I would also like to acknowledge Drs. Neal Swerdlow, Vaishali Bakshi and Andrew Chambers for their additional support in experimental design and implementation of my co-morbidity of sensorimotor gating deficits and methamphetamine reward model. Furthermore, I would like to thank past and present members of the Napier Laboratory. You all have been outstanding mentors, collaborators and friends. Finally, I would like to extend my heartfelt gratitude and love to my family, specifically my mother Anne Herrold and father Robert Herrold. Also, I would like to thank my significant other, John McDaid, who has given me endless support and believed in me always.

The work in this dissertation is supported by USPHS grants DA015760 to TCN and DA023306 to AAH and TCN, and the Loyola University Chicago Graduate School.

To my parents Anne Herrold and Robert Herrold

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LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
Amph	amphetamine
BS^3	bis(sulfosuccinimidyl)suberate
CDPPB	3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide
Coch	cochlear nucleus
CPP	conditioned place preference
CREB	cAMP-response element binding protein
CS	conditioned stimulus
CSF	cerebral spinal fluid
DA	dopaminergic
DAT	dopamine transporter
DSM-IV	*Diagnostic and Statistical Manual of Mental Disorders, 4th Edition*
EC_{50}	half maximal effective concentration
ED_{50}	half maximal effective dose
GABA	gamma-aminobutyric acid-ergic
GAD_{67}	glutamic acid decarboxylase 67
Glu	glutamate
GPCRs	G-protein coupled receptors
Hipp	hippocampus

I	intracellular component
IC	inferior colliculus
i.c.v.	intracerebroventricular
IUPAC	international union of pure and applied chemistry
i.p.	intraperitoneally
JNJ16259685	(3,4-dihydro-2H-pyranol[2,3]b quinolin-7-yl)(cis methoxycylohexyl)methanone
LTP	long term potentiation
MDT	medial dorsal thalamus
Meth	methamphetamine
mGluRs	metabotropic glutamate receptors
mGluR1	metabotropic glutamate receptor group I subtype 1
mGluR5	metabotropic glutmate receptor group I subtype 5
MOR	morphine
MPEP	2-methyl-6-(phenylethynyl)-pyridine
mPFC	medial prefrontal cortex
MTEP	3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine
NET	norepinephrine transporter
NAc	nucleus accumbens
NAM	negative allosteric modulator
NMDAR	N-methyl-D-aspartic acid receptor
Non-Xlink	without cross-linking
NVHL	neonatal ventral hippocampal lesion
PAM	positive allosteric modulator

PCP	phencyclidine
PFC	prefrontal cortex
PLC	phospholipase C
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PnC	nucleus reticularis pontis
PI	phosphoinositide
PPI	prepulse inhibition
PPTg	pedunculopontine tegmental nucleus
rm	repeated measures
sal	saline
SEM	standard error of the mean
SERT	serotonin transporter
S	surface component
SC	superior colliculus
s.c.	subcutaneously
S/I	surface to intracellular
STEP	striatal enriched protein tyrosine phosphatase
$STEP_{61}$	striatal enriched protein tyrosine phosphatase isoform 61
Total	total protein
UCS	unconditioned stimulus
VMAT	vesicle monoamine transporter
VP	ventral pallidum
VTA	ventral tegmental area

Xlink	cross-link

CHAPTER I

INTRODUCTION

Methamphetamine (Meth) is a potent, widely-used stimulant and its use is met with high rates of relapse without an effective, FDA-approved pharmacotherapy (Brecht et al. 2000;Elkashef et al. 2008;United Nations Office on Drugs and crime 2009). One factor that contributes to craving and relapse is exposure to environmental or contextual cues associated with previous Meth use (Hartz et al. 2001;O'Brien et al. 1992;Tolliver et al. 2010). With repeated Meth use, the rewarding properties of the drug become associated with the environmental cues in which they are taken. These contextual cues then become extremely salient to the Meth user. This associative learning can be studied in the laboratory with humans and rodents using conditioned place preference (CPP), which employs classical conditioning to measure the rewarding properties of abused drugs (Childs and deWit H. 2009;Tzschentke 1998;Tzschentke 2007). Another way to assess Meth-induced brain adaptations following repeated Meth use is the enhanced motor activity (termed motor sensitization) that is elicited with subsequent drug injections. This behavior endures for long periods of time after cessation from drug administration (McDaid et al. 2007). It is hypothesized that the neuronal adaptations associated with this motor sensitization model aspects of the brain changes that reflect drug craving (Robinson and Berridge 1993).

The current dissertation project utilizes Meth-induced CPP and motor sensitization to elucidate the neuronal underpinnings of addiction. Repeated drug stimuli are hypothesized to "hijack" normal neuronal mechanisms of learning and memory processes, resulting in abnormal, addictive behaviors (Kelley 2004). The glutamate transmitter system is an important player for learning and memory processing as well as stimulant addiction. Glutamate activates both ionotropic and metabotropic receptors. Ionotropic receptors rapidly increase neuronal spiking, whereas metabotropic glutamatergic receptors (mGluRs) modulate and fine tune neuronal excitability. mGluRs are divided into three groups based on pharmacology, sequence homology and associated signaling mechanisms (Conn and Pin 1997). The group I subtype 5 receptor (mGluR5) is highly expressed in brain regions important for stimulant reward (Lu et al. 1999;Testa et al. 1995). The mGluR5 is important for several phases of behaviors induced by amphetamines, including Meth, such as acquisition (Miyatake et al. 2005;Osborne and Olive 2008) and expression (Gass et al. 2009;Herzig et al. 2005) of associative learning. However, the role of these receptors in the maintenance of Meth-induced reward remains unknown. This is an important clinical issue, as treatment strategies need to be effective following the molecular adaptations and behavioral changes have occurred. Withdrawal time after the last drug administration influences drug-induced behaviors as well as underlying molecular adaptations. For example, rodent models of addiction including opiate-induced conditioned place preference and stimulant- induced self-administration demonstrate that cue-induced drug seeking can "incubate"

over a time frame of two weeks to three months after the cessation of drug treatment (Li et al. 2008;Lu et al. 2004a;Lu et al. 2004b). Furthermore, the glutamate receptor systems, including the mGluR5, also are up-regulated in reward-related brain regions following extended (but not short term) withdrawal from cocaine administration (Ghasemzadeh et al. 2009a;Ghasemzadeh et al. 2009b). Therefore, we sought to determine if the mGluR5 was important for long-term maintenance of Meth-induced associative learning.

Substance use disorders occur with a higher frequency in the schizophrenia patient population than in the general United States population (Compton et al. 2005). Moreover, the use of amphetamines exacerbates psychosis in schizophrenia patients (Angrist et al. 1980;Janowsky and Davis 1976). Developmental rodent models of schizophrenia (e.g., isolation rearing and neonatal ventral hippocampal lesions) demonstrate an enhanced sensitivity to stimulant-induced motor sensitization (Chambers and Taylor 2004;Dai et al. 2004) and self-administration (Brady et al. 2008;Chambers and Self 2002), but a deficit in stimulant-induced associative learning that are likely due to cognitive deficits incurred with this disorder (Le et al. 2002;Wongwitdecha and Marsden 1995). These observations likely reflect the fact that the neurocircuitry implicated in schizophrenia overlaps with that targeted by stimulants (Pierce and Kalivas 1997;Swerdlow et al. 2001). Therefore, we hypothesized that deficits associated with schizophrenia, such as sensorimotor gating, would negatively correlate with that of Meth-induced associative learning (i.e., greater deficits in sensorimotor gating would correlate with less preference for the context

associated with the rewarding properties of Meth). Thus, we used both pharmacological and developmental (i.e. isolation rearing) models of schizophrenia in conjunction with Meth-induced CPP to model the co-morbid condition of the schizophrenia individual with stimulant use disorder. We further sought to determine the effects of augmenting mGluR5 signaling on the different phases of Meth-induced CPP and motor sensitization in isolation reared rats (i.e., development and expression).

The ***overall goal*** of this dissertation is to expand our knowledge of the role of the mGluR5 system in Meth addiction and in the co-morbidity of schizophrenia and Meth substance use disorder. This project is subdivided into two major parts with individual, yet related hypotheses. ***Part 1*** was designed to determine if the mGluR5 is necessary for the maintenance of Meth-induced associative learning, and if this was associated with an up-regulation of these receptors in reward-related brain regions. ***Part 2*** was designed to ascertain if deficits associated with schizophrenia are directly correlated with Meth-induced associative learning and if mGluR5 receptor activation enhances Meth-induced CPP in a developmental rodent model of schizophrenia.

CHAPTER II

LITERATURE REVIEW

Methamphetamine substance use disorder and rodent behavioral models

Methamphetamine (Meth) was given the title "America's most dangerous drug" by Newsweek Magazine in August 2005 by David J. Jefferson. In the same year, it was estimated that Meth use resulted in 900 deaths and the loss of 44,000 quality of life years (Nicosia et al. 2009). The estimated total cost to the United States in 2005, including factors such as drug treatment, criminal justice, and child endangerment reached $23.5 billion (Nicosia et al. 2009). Though there has been a decline in prevalence of Meth use since 2005, Meth remains the third most widely abused drug in the world (United Nations Office on Drugs and crime 2009). Meth use is a problem of grave concern with high health, social, and economic costs that merit scientific investigation.

Meth is a powerfully rewarding substance due to its ability to greatly increase brain monoamines. Meth and other amphetamines act as a substrate for plasma membrane monoamine transporters including dopamine (DAT), serotonin (SERT) and norepinephrine (NET), thereby inhibiting the reuptake of these transmitters (Bonisch 1984;Fleckenstein et al. 1999;Han and Gu 2006;Haughey et al. 2000;Jones et al. 1998;Liang and Rutledge 1982;Zaczek et al. 1991). Amphetamines bind to vesicle monoamine transporters (VMAT) located on the monoamine containing synaptic vesicles (Partilla et al. 2006;Peter et al. 1994). VMATs use secondary active transport via a coupled proton pump to transport monoamine transmitters into the vesicle (Schuldiner et al. 1998). Amphetamines are weak bases, and upon entering the vesicle amphetamines increase the vesicular pH and reduce the vesicular/cytoplamic pH gradient, thus reducing the energy barrier for sequestered transmitter to be released into the cytosol (Sulzer et al. 1993;Sulzer et al. 1995;Sulzer and Rayport 1990). This results in a decrease in monoamine transmitter in the vesicle, an increase in transmitter in the cytosol, and, through reverse transport at the monoamine transporters, an increase in transmitter release to the extracellular synaptic cleft (Sulzer et al. 1995). Amphetamines also act as competitive monoamine oxidase inhibitors, which are yet another means of increasing monoamine concentration within the cell by hindering catabolism (Mantle et al. 1976;Scorza et al. 1997). Thus, through its action at monoamine transporters, VMATs, and monoamine oxidase, Meth acts to increase monoamine neurotransmitters released into the synaptic cleft.

Meth (international union of pure and applied chemistry (IUPAC) nomenclature = N-methyl-1-phenyl-propan-2-amine) differentiates itself from amphetamine (Amph, IUPAC nomenclature = (±)-1-phenyl-propan-2-amine) in having an additional methyl group that allows for greater brain penetration and more persistent effects (NIDA Research Report Methamphetamine Abuse and Addiction 2006). Moreover, the rates of illicit Meth use are reported to be higher than that of Amph (Colliver 2006). In humans, the half life of Meth is approximately 10 hr (Cook et al. 1993;Harris et al. 2003;Newton et al. 2005) and the subjective "high" occurs rapidly after approximately 10min (Perez-Reyes et al. 1991). The subjective effects of Meth in humans include arousal, euphoria, relaxation, anxiety, talkativeness, paranoia, and hallucinations (Bell 1973;Martin et al. 1971). Meth is a sympathomimetic that produces physiological effects including increases in heart rate, blood pressure, and respiration rate (Ho et al. 2009;Martin et al. 1971) and a propensity for stroke (Perez, Jr. et al. 1999;Rothrock et al. 1988).

The laboratory rat is often used to model human Meth addiction, and the pharmacodynamics of Meth in the rat emulates the human; however, the pharmacokinetics of Meth is quite different. In the rat, the half life is of Meth is 70min after an intravenous administration (Cho et al. 2001;Riviere et al. 1999). After intraperitoneal (i.p.) administration, peak locomotor activity following an acute, low dose of Meth (1-2mg/kg) occurs at approximately 30min (Shoblock et al. 2003). The Napier laboratory reports similar peak motor effects for an acute dose of subcutaneously (s.c.) administered Meth (1mg/kg) and that this motor

activity is enhanced following repeated treatment (2.5mg/kg, s.c. per day for 5 days) (McDaid et al. 2007). These pharmacokinetic factors must be considered in experimental design using rodent models of addiction.

The rewarding subjective effects of Meth can be associated with the context in which they are administered. Presentation of cues associated with drugs, such as pictures of people taking drugs or drug paraphernalia produce an enhanced subjective and physiological state similar to that experienced with drug taking (Carter and Tiffany 1999;Tolliver et al. 2010). Moreover, human imaging data demonstrate that exposure to drug-related cues can enhance activity in reward-related brain regions of stimulant addicts (Breiter et al. 1997;Childress et al. 1999;Grant et al. 1996;Kilts et al. 2004;Maas et al. 1998), and lead to craving and relapse in the withdrawn addict. Newton and colleagues recently reported that 15% of Meth-dependent individuals surveyed relapse due to cravings and 44% stated that they relapsed because they wanted “to get high” (Newton et al. 2009). Though drug craving may not be the only factor contributing to relapse, craving in Meth-dependent individuals has been shown to predict Meth use (Hartz et al. 2001). The presentation of drug cues to stimulant withdrawn addicts increases measures of craving and drug-taking behavior in a clinical laboratory setting (Hogarth et al. 2010;Mucha et al. 1998;Panlilio et al. 2005;Tolliver et al. 2010). Cues associated with stimulant use produce a strong psychological and physiological response in stimulant abusers that can lead to craving and relapse. Therefore, uncoupling these contextual drug cues with the

rewarding properties of Meth may provide an important means of relapse prevention in stimulant use disorders.

A behavioral model that measures the association between drug-paired cues and the rewarding effects of abused drugs is conditioned place preference (CPP). CPP implements classical or Pavlovian conditioning (Pavlov 1927). In this paradigm, the unconditioned stimulus (UCS; e.g., Meth) is paired with a neutral environmental context, termed the conditioned stimulus (CS), during a process called conditioning. After conditioning, the subject demonstrates that the enhanced salient properties of the UCS are transferred to the CS, and in CPP this process is demonstrated by the subject preferring the drug-paired context in the absence of the UCS. This is termed expression of CPP. CPP is reliably used to measure the rewarding effects of Meth as well as Amph and other abused substances in laboratory rodents (Spyraki et al. 1982;Tzschentke 1998;Tzschentke 2007). Recently, Childs and deWit report that this behavioral paradigm can be directly tested in humans using Amph as the UCS (Childs and deWit H. 2009). Therefore, the CPP behavioral model provides a means for assessing the association between rewarding effects of drugs and conditioned contextual cues that is relevant in humans.

Another index of brain plasticity that occurs with repeated drug administration, aside from CPP, is the progressive enhancement in motor activity, termed motor sensitization. Neuronal structures upon which psychostimulants and other drugs of abuse act adapt to produce an enhanced response with repeated exposure to the drug stimulus (Stewart and Badiani

1993). It is hypothesized that the neuronal adaptations associated with motor sensitization may model certain aspects of the brain changes that are associated with Meth abuse in humans (Robinson and Berridge 1993;Stewart and Badiani 1993). The sensitizing effect of Amph, for example, is so powerful that a single administration of the drug can enhance responding to subsequent Amph administration, and these sensitized behaviors evoked by Amph become more pronounced even after long periods of withdrawal from repeated exposure (e.g., 3 weeks) (Vanderschuren et al. 1999). Sensitization to the effects of Amph also occur in humans after repeated, intermittent administration including enhancements in Amph-induced euphoria, energy level, and talkativeness (Strakowski et al. 1996;Strakowski et al. 2001;Strakowski and Sax 1998). Repeated Amph and Meth induce psychotic effects similar to those reported in schizophrenia (Angrist and Gershon 1970;Bell 1973;Griffith et al. 1972) which also demonstrates persistent sensitization-like properties (Robinson and Becker 1986;Schmidt and Beninger 2006;Yui et al. 1999). Our laboratory and others have demonstrated that Meth and Amph can differentially induce CPP and motor sensitization behaviors in rodents depending on factors such as drug dosage (Itzhak et al. 2002;Shen et al. 2006), rodent age (Belluzzi et al. 2004), and rodent strain (Kosten et al. 1994). These reports suggest that the two behaviors may model different aspects of human drug addiction.

Relapse rates for Meth addicts undergoing behavioral/cognitive recovery programs reach approximately 50-60% (Brecht et al. 2000;McLellan et al. 2000), and there is currently no FDA-approved pharmacotherapy for Meth use disorders

(Elkashef et al. 2008). Therefore, the treatment of Meth use disorders represents an unmet need for which the development of more effective pharmacotherapy merits investigation. The current studies implemented CPP and motor sensitization in order to identify potential therapeutic targets for addiction therapy.

Dual diagnosis of substance use disorders and schizophrenia: clinical scenario and rodent behavioral models

A recent clinical assessment determined that 25% of schizophrenia patients also meet criteria for psychostimulant abuse or dependence (Compton et al. 2005), a frequency that is considerably higher than the 5% of the general United States population reported to have used or abused Meth in 2004 (http://www.drugabusestatistics.samhsa.gov/2k5/meth/meth.htm). Dual diagnosis schizophrenia patients show an increased incidence of violence (Buckley et al. 2004), as well as greater housing, economic, and health care access problems (Compton et al. 2005). Case studies demonstrate that the atypical antidepressant, olanzapine, can reduce psychotic symptoms induced by Meth abuse, but it remains unclear if this treatment reduces relapse to Meth abuse in the stimulant abstinent co-morbid patient (Misra et al. 2000). Fifty-five percent of schizophrenia patients who are medicated with antipsychotics are substance abusers (Swofford et al. 2000), indicating that blocking dopamine transmission is insufficient to alter stimulant abuse. There clearly is an unmet

need for an effective pharmacotherapy for the dual diagnosis patient. This project is predicated on the concept that a better understanding of the behavioral profile of dual diagnosis will shed light on the neurobiological underpinnings that overlap between stimulant use disorders and schizophrenia.

Schizophrenia is described by the *Diagnostic and Statistical Manual of Mental Disorders, 4th Edition* (*DSM-IV*) as a mixture of characteristic positive and negative "signs and symptoms associated with marked social or occupational dysfunction." Positive symptoms include "distortions in thought content (delusions), perception (hallucinations), language and thought process (disorganized speech), and self-monitoring of behavior (grossly disorganized or catatonic behavior)" while negative symptoms include "restrictions in the range and intensity of emotional expression (affective flattening), in the fluency and productivity of thought and speech (alogia), and in the initiation of goal-directed behavior (avolition)" (American Psychiatric Association 2000). As stated in the previous section, repeated use of amphetamines results in psychotic symptoms akin to schizophrenia in humans, including cognitive dysfunction, delusions, and hallucinations (Harris and Batki 2000;McKetin et al. 2006;Scott et al. 2007). Though the *DSM-IV* classifies substance-induced psychotic disorders separately, there is evidence to suggest that use of amphetamines results in an augmentation of symptoms in schizophrenia patients (Angrist et al. 1980;Janowsky and Davis 1976). The striking similarity between clinical presentations of schizophrenia and psychosis induced by amphetamines lends credence to the idea that there is an overlapping neurobiological function between the two disorders. One

behavioral feature that occurs with both schizophrenia patients and administration of amphetamines is a sensorimotor gating deficit that indicates cognitive fragmentation (Braff et al. 1978;Grillon et al. 1992). This can be measured in both humans and rodents with the prepulse inhibition (PPI) deficits of the acoustic startle response (Braff et al. 2001;Swerdlow and Geyer 1998). PPI is defined as the natural inhibitory response that occurs when a startling stimulus is preceded by a weaker stimulus (Graham 1975;Hoffman and Searle 1968;Ison et al. 1973;Swerdlow and Geyer 1998)(Fig. 1). Recent studies report that sensitizing treatment regimens of Amph disrupt PPI and Amph-induced PPI deficits in laboratory rats can model of aspects of human schizophrenia (Peleg-Raibstein et al. 2008;Tenn et al. 2005). Assays of brain chemistry and anatomy in Amph-treated rats demonstrate further similarities to the schizophrenia brain state such as a decrease in the enzyme glutamic acid decarboxylase 67 (GAD_{67}) in the prefrontal cortex, hippocampus and thalamus (Akbarian et al. 1995;Heckers et al. 2002;Peleg-Raibstein et al. 2008;Perry et al. 1979;Volk et al. 2000). Therefore, further study into the behavioral outcomes induced by repeated Amph such as sensorimotor gating deficits and reward-mediated behaviors will help elucidate neurobiological underpennings of, and identify therapeutic targets for, the dually diagnosed patient.

Early stressors in development of laboratory rodents such as maternal and social deprivation (termed isolation rearing), produce behavioral, neurochemical, and morphological adaptations that result in a phenotype that resembles aspects of schizophrenia. In this model, rats are separated from their mother

approximately one day post-weaning (post-natal day 21) and housed individually until adulthood with minimal handling by the experimenter (Einon and Morgan 1977;Varty et al. 1999). Sensorimotor gating deficits are observed in isolation-reared rats (Geyer et al. 1993). Isolation rearing-induced PPI deficits are reversed by antipsychotic medications used to treat symptoms in schizophrenia patients, giving predictive validity of isolation rearing as a model for schizophrenia (Bakshi et al. 1998). Furthermore, there is evidence of decreases in volume of the frontal cortex in both isolation reared rodents (Day-Wilson et al. 2006) and schizophrenia patients (Benes et al. 1991), which indicates similar morphological alterations. Isolation rearing provides an ideal non-pharmacological means of assessing sensorimotor gating deficits associated with schizophrenia in the adult rat.

Other rodent models of schizophrenia demonstrate enhanced sensitivity to psychostimulants; a phenomenon that is known to occur in humans (Angrist et al. 1980;Janowsky and Davis 1976). The model predominantly used in these studies is the neonatal ventral hippocampal lesion (NVHL). In this model, bilateral excitotoxic lesions are produced in the ventral hippocampus on post natal day seven which disrupts the hippocampal connection to the prefrontal cortex. This results in behavioral and neurochemical properties in rats that are similar to features presented by human patients with schizophrenia (Lipska et al. 1993;Lipska et al. 1995). The NVHL rodents show enhanced motor sensitization to nicotine and cocaine compared to sham lesioned counterparts. The acquisition of both cocaine (Chambers and Self 2002) and Meth self-administration (Brady

et al. 2008) is also enhanced in NVHL rats. Furthermore, NVHL lesioned rodents displayed enhanced motivation to work for Meth administration when the schedule of reinforcement was altered to a progressive ratio in the self-administration paradigm (Brady et al. 2008). Like the NVHL rats, isolation-reared rodents also demonstrate enhanced motor sensitization to Meth (Dai et al. 2004), cocaine and amphetamine (Lipska et al. 1993) as well as enhanced responding in a progressive ratio for both cocaine and amphetamine (Smith et al. 1997). Taken together, motor sensitization and self-administration studies indicate that the NVHL and isolation rearing rodent models of schizophrenia are more sensitive to the locomotor stimulating and reinforcing effects of stimulants, which may reflect positive symptoms associated with schizophrenia. However, these developmental rodent schizophrenia models demonstrate a diminished response to the rewarding properties of stimulant drugs, which could reflect negative symptoms associated with schizophrenia such as anhedonia and cognitive dysfunction. For example, in CPP paradigms, isolation reared rats fail to demonstrate a significant preference for environmental context associated with Amph (Wongwitdecha and Marsden 1995) and the opiate morphine (Wongwitdecha and Marsden 1996). Rats with the NVHL lesion also showed blunted CPP induced by Amph or the natural reward saccharin (Le et al. 2002). Overall, in developmental rodent models of schizophrenia there is a disparity in the rewarding vs. reinforcing properties of stimulants. Therefore, one objective of the current dissertation project was to investigate how sensorimotor gating

deficits associated with different rodent models of schizophrenia correlate with rewarding and incentive motivational properties of Meth.

Glutamate transmission in addiction and schizophrenia

The reinforcing properties of abused stimulants and the neuropathology of schizophrenia are both associated with hyperactivity of the mesolimbic dopamine system (see reviews (Carlsson 1988;Wise and Rompre 1989)). Most abused drugs increase extracellular dopamine concentrations in the nucleus accumbens (NAc) (Di Chiara and Imperato 1988), which receives dopaminergic input from the ventral tegmental area (VTA) (Swanson 1982). Amphetamines also increase extracellular glutamate concentrations in the NAc and other regions where VTA dopamine projections terminate such as the mPFC and ventral pallidum (VP) (Chen et al. 2001;Shoblock et al. 2003;Xue et al. 1996). The mPFC provides glutamatergic innervations to both the NAc and VP (Christie et al. 1985;Fuller et al. 1987;Sesack et al. 1989) as well as the VTA (Sesack and Pickel 1992). The VP and NAc have reciprocal GABAergic connections (Jones and Mogenson 1980;Mogenson et al. 1983;Walaas and Fonnum 1979;Zahm et al. 1985). The NAc receives glutamatergic projections from the hippocampus and amygdala (McDonald 1991;Meredith et al. 1990). The amygdala also sends a glutamatergic projection to the VP (Carnes et al. 1990;Fuller et al. 1987;Russchen and Price 1984). The current literature review will focus on the mPFC, NAc and VP as these

regions are critical players in stimulant-induced motor sensitization, conditioned reward, schizophrenia and sensorimotor gating (see Fig. 2 for circuitry diagram).

Glutamate alterations specific to motor sensitization

Rodent studies demonstrate that mPFC, NAc and VP brain structures are involved in different phases of motor sensitization. Initial studies revealed that lesions of the mPFC reduce induction of Amph-induced motor sensitization (Bjijou et al. 2002;Cador et al. 1999;Wolf et al. 1995;Wolf and Xue 1999) but not the expression of this behavior (Li and Wolf 1997). Tzschentke later revealed, however, that mPFC lesions do not affect the development of Amph-induced motor sensitization (Tzschentke and Schmidt 1999;Tzschentke and Schmidt 2000). These disparate findings may be due to differences in environmental conditions associated with Amph administration and lesion extent. However, the literature is in agreement that mPFC lesions disrupt both the development and expression of cocaine-induced motor sensitization (Li et al. 1999;Pierce et al. 1998;Tzschentke and Schmidt 1999;Tzschentke and Schmidt 2000). To date, there is no study employing lesions of the mPFC to determine its effects on Meth-induced motor sensitization. It is known that extracellular glutamate levels in the mPFC are differentially altered by systemic administration of Amph *vs.* Meth. That is, there is an increase in mPFC glutamate levels after Meth (2mg/kg) injections peaking approximately 100min post-injection but no changes occur in mPFC glutamate levels after Amph (2mg/kg) administration (Shoblock et al. 2003). Preliminary work from the Szumlinski laboratory demonstrated a

reduction in mPFC extracellular glutamate levels three weeks after repeated Meth (Lominac and Szumlinski 2008). However, direct infusion of Amph into the mPFC results in a dose-dependent increase in glutamate levels in the same brain region (Del Arco et al. 1998). The neuronal firing response of mPFC neurons to local, microiontophoretic application of glutamate is also enhanced in Amph-sensitized rats (Peterson et al. 2000). These studies implicate the mPFC as an important structure in the development of stimulant-induced motor sensitization and that neurons of this region are hyper-responsive to glutamate during withdrawal from repeated exposure to stimulants.

The NAc is a brain region classically considered important for the expression of stimulant-induced motor sensitization (see reviews (Pierce and Kalivas 1997;Vanderschuren and Kalivas 2000). Glutamate neurotransmission is important in this role, since local injection of NMDA receptor antagonist (AP-5) into the core region of the NAc blocks cocaine-induced motor sensitization (Pulvirenti et al. 1994). Furthermore, increases in extracellular glutamate occur in the NAc only in rats sensitized to cocaine subsequent to an additional cocaine injection, and this effect is specific to the core subregion of the NAc (Pierce et al. 1996). Pierce and Kalivas also demonstrated that local injection of AMPA into the core but not the shell subregion of the NAc enhanced locomotor effects in cocaine-sensitized rats (Pierce et al. 1996). However, NAc neurons are less sensitive to locally applied glutamate three days after administration of a sensitizing regimen of cocaine or Amph, perhaps due to a shorter withdrawal period (White et al. 1995). These findings converge to show the importance of

glutamate in the NAc core to the expression of stimulant-induced motor sensitization.

An output structure of the NAc, the VP, may play a role in the development and expression of stimulant-induced motor sensitization. Local injection of Amph into the VP increases motor activity but to a lesser extent than local NAc application (Fletcher et al. 1998). Blockade of AMPA/kainate receptors in the VP also reduces acute Amph-induced hyperactivity (Willins et al. 1992). The expression, however, of Amph-induced motor sensitization is reduced by intra-VP administration of NMDA receptor antagonists (Chen et al. 2001). Furthermore, a challenge administration of Amph given 10-14 days after repeated Amph treatments increases extracellular glutamate levels in the VP (Chen et al. 2001). Our lab has demonstrated that the there is an increase in neuronal response of VP neurons three days following repeated, sensitizing cocaine administration (McDaid et al. 2005). These studies demonstrate that the VP glutamate system is involved in the acute hyper-motoric effects of stimulants as well as in the expression of stimulant-induced motor sensitization.

Neuronal activation and glutamatergic alterations following stimulant-induced associative learning

The complex neuronal processes involved with addiction are thought to involve hijacking normal components of learning and memory. The PFC, important for executive function and control of memory maintenance (Goldstein and Volkow 2002;MacDonald, III et al. 2000), has decreased activation in Meth-

addicted humans performing cognitive tasks (Paulus et al. 2003;Salo et al. 2009). However, the PFC becomes highly activated with the presentation of stimulant-related cues in subjects with stimulant use disorders (Grant et al. 1996;Hester and Garavan 2009;Maas et al. 1998). Meth conditioned rats given a priming injection of Meth and re-exposed to Meth-cues demonstrate enhanced extracellular mPFC glutamate levels compared to rats that were not primed with Meth (Qi et al. 2009). These data indicate that the mPFC is sensitive to Meth and Meth-related cues. The mPFC projects to the NAc, and recently both of these regions were proven to be critical for cue- and Meth-induced reinstatement of drug-seeking behavior in rodents (Rocha and Kalivas 2010). The NAc is a central component of the reinforcing, rewarding and hyper-motoric effects of stimulants (Bozarth and Wise 1981;Swerdlow et al. 1986;Wise and Bozarth 1985). While the NAc is a central mediator of the reinforcing effects of stimulants, it may not be as critical to the learning or craving associated with the drug and drug cues, since cortical regions but not subcortical structures such as the NAc are metabolically activated in the presence of cocaine cues (Grant et al. 1996) but also see (Breiter et al. 1997). In rodent studies of cellular activation, there is an increase in fos expression after Meth administration in the NAc (Lee et al. 2000). However, there is an increase in fos expression in the mPFC but not in the NAc in rodents responding to cocaine cues (Brown et al. 1992;Mattson and Morrell 2005). However, Rhodes *et al.* found an increase in c-Fos expression in the NAc shell as well as in the PFC in response to Meth-associated cues (Rhodes et al. 2005). Based on behavioral and cellular activity data, the mPFC and NAc play important

roles in response to stimulant cues. While imaging data suggests that the mPFC might be more sensitive to drug-related cues, the resolution of these procedures may make discerning activation in the NAc or VP more difficult.

The VP plays a critical role in integration of neuronal signaling to produce motivated motor behavior (Mogenson and Yang 1991). VP neurons encode information relevant to incentive properties of cues that predict reward, which is enhanced subsequent to Amph sensitization (Tindell et al. 2005). NMDA receptors in the VP are necessary for the development but not expression of amphetamine-induced CPP (Hiroi and White 1993). Work conducted in the Napier lab revealed an increase in the stable form of the FosB immediate early gene family, ΔFosB, that persists following a sensitizing regimen of Meth in the VP (McDaid et al. 2006b). Biochemical and electrophysiological data suggest that VP neurons are activated by stimulants and related cues.

In summary, the mPFC, NAc, and VP all demonstrate cellular or region activation in response to stimulant administration, and these responses are enhanced following repeated exposure. The mPFC is hyper-activated in response to the presentation of stimulant-related cues in human addicts.

Glutamate alterations associated with schizophrenia and sensorimotor gating deficits

The glutamate hypothesis of schizophrenia was born out of clinical evidence that individuals taking phencyclidine (PCP), the ionotropic glutamate NMDA receptor open channel blocker, experienced a psychotic state similar to

that of schizophrenia (Allen and Young 1978;Fauman et al. 1976;Rainey, Jr. and Crowder 1975), and that PCP exacerbated illness in schizophrenia patients (Itil et al. 1967). PCP can also induce sensorimotor gating deficits in rodents and non-human primates (Linn and Javitt 2001;Martinez et al. 1999) that are reversed by antipsychotic medications (Bakshi et al. 1994;Bakshi and Geyer 1995;Linn et al. 2003). Levels of glutamate are lower in the cerebral spinal fluid (CSF) of schizophrenia patients than in individuals without the illness (Kim et al. 1980). There is pre-clinical and clinical evidence that schizophrenia is not merely a disorder of a hyperactive dopamine system but also involves glutamatergic dysregulation.

Limbic circuitry associated with reward innervates the brain stem circuitry that mediates the acoustic startle response. Briefly, auditory stimuli are received by the inferior colliculus (IC) via the cochlear nucleus (Coch) and relayed to the superior colliculus (SC) and pedunculopontine tegmental nucleus (PPTg). The PPTg sends descending cholinergic projections to the nucleus reticularis pontis (PnC) of the primary startle pathway as well as sending excitatory ascending projections to the thalamus and dopaminergic nuclei of the reward pathway such as the VTA. The PnC can also be directly activated by auditory stimuli and sends projections to spinal motor neurons eliciting a startle response (see Fig. 2, and reviews, (Davis et al. 1982;Fendt et al. 2001;Swerdlow et al. 1992). The limbic reward circuitry (discussed above) modulates the PPI circuit predominantly at the level of the PPTg via GABAergic projections from the VP and NAc (Chivileva and Gorbachevskaya 2008;Haber et al. 1990). I will limit

my review of brain regions involved with the modulation of PPI to those discussed previously, the mPFC, NAc and VP (see Fig. 2 for diagram of circuitry).

Post-mortem morphological studies identify a decreased neuronal density in the mPFC of schizophrenia patients (Benes et al. 1986). It was further determined that this lower density was due to a loss of small, likely GABAergic interneurons and that levels of larger pyramidal neurons were generally unaltered (Benes et al. 1991). This morphological alteration may contribute to the sensorimotor gating deficits in schizophrenia patients since intra-mPFC administration of picrotoxin, the $GABA_A$ receptor antagonist, disrupts PPI in rodents (Japha and Koch 1999). A reduction in GABAergic inhibition in the mPFC would result in an increase in glutamate release in the VTA leading to an increase in dopamine release in the NAc (see Fig. 2 for circuitry). The mechanism by which PPI is disrupted in the mPFC is likely associated with a loss of dopaminergic inhibition of glutamate neurons since lesions of dopamine terminals reduce PPI and this effect is reversed by administration of the antipsychotic haloperidol that has a high affinity for dopamine D2 receptors (Bubser and Koch 1994;Koch and Bubser 1994). The deficits in PPI induced by local injections of picrotoxin into the mPFC are also reversed by haloperidol, further substantiating this claim (Japha and Koch 1999). Therefore a loss of inhibition of in the mPFC via both GABAergic and dopaminergic means may lead to an increase in NAc dopamine resulting in PPI deficits associated with schizophrenia. Isolation-reared rodents that displayed PPI deficits have reduced mPFC volume (neuronal number was unaltered), which further validates the role

of mPFC in sensorimotor gating deficits associated with schizophrenia as well as the utility of the isolation-rearing model (Day-Wilson et al. 2006). Other studies reveal a disconnection in PFC neurons (Bagorda et al. 2006;Witte et al. 2007) and a reduction in metabotropic glutamate receptors in isolation reared rodents (Melendez et al. 2004). Therefore, the mPFC is key in sensorimotor gating deficits associated with schizophrenia.

The NAc receives glutamatergic projections from the mPFC, hippocampus, amygdala, and dopaminergic innervation from the VTA. The NAc, thus, is an important site for glutamate and dopamine transmitter interaction. Zhang and colleagues report a close correlation between Amph-induced deficits in sensorimotor gating and increases in extracellular dopamine levels in the NAc (Zhang et al. 2000). Local injection of Amph into the NAc disrupts PPI (Swerdlow et al. 2007;Wan and Swerdlow 1996), an effect that is reduced by the co-administration of ionotropic AMPA receptor antagonist, CNQX (Wan and Swerdlow 1996). Furthermore, intra-NAc administration of AMPA or NMDA alone decreases PPI (Reijmers et al. 1995;Wan et al. 1995). Lesions of dopamine terminals in the NAc and administration of haloperidol reverse AMPA-induced disruptions of PPI (Wan et al. 1995). Clearly, the NAc is a region critical for the dopamine-induced effects on sensorimotor gating that are also affected by glutamatergic transmission.

The NAc and VP are connected via reciprocal GABAergic projections and both regions innervate the PPTg (Haber et al. 1990;Heimer et al. 1991;Parent et al. 1999). Volume loss in the VP is reported in post-mortem brain tissue from

schizophrenia patients (Bogerts et al. 1985). Lesions of the VP as well as GABA agonist application in this region reveal that it is a critical mediator in the sensorimotor gating deficits associated with excess dopamine in the NAc (Kretschmer and Koch 1998;Swerdlow et al. 1990). Further evidence for the role of VP GABA transmission in regulation of dopamine induced PPI is demonstrated by dopamine receptor (D3/D2) agonist quinelorane inducing increased extracellular GABA levels in the VP as well as reducing PPI in rodents (Qu et al. 2008). The VP works to integrate dopamine- and GABA-mediated signaling to modulate sensorimotor gating behavior.

Involvement of the metabotropic glutamate receptor group I, subtype 5 (mGluR5) in reward-mediated behaviors

Glutamate activates ionotropic glutamate receptors and metabotropic glutamate receptors. Metabotropic glutamate receptors (mGluRs) are classified into three different groups based on sequence homology, pharmacology and associated signaling cascades with which they are coupled (Nakanishi 1992). The mGluRs have seven transmembrane domains, a large extracellular amino-terminus, an intracellular carboxy-terminus, and are members of the family 3/C G-protein coupled receptors (GPCRs) (see for review (Conn and Pin 1997;Hermans and Challiss 2001;Pin et al. 2003)). The group I subtype 1 receptor (mGluR1) was the first cloned mGluR (Houamed et al. 1991;Masu et al. 1991) and subsequent cloning of group I subtype 5 metabotropic glutamate

receptor (mGluR5) determined that the two receptors share approximately 87% sequence homology (Abe et al. 1992). The mGluR5 also exists in a covalent, disulfide-linked homodimer in non-reducing conditions (Romano et al. 1996) but also see evidence for non-covalent dimerization (Romano et al. 2001). The extracellular amino-terminal region of the group I mGluR homodimer forms a venus-flytrap-shaped region where agonist binding occurs (Kunishima et al. 2000). Also, mGluR5 demonstrates constitutive activity, and both the extracellular amino terminal (Muhlemann et al. 2005) and the seven-transmembrane domain play roles in this receptor behavior (Goudet et al. 2004). The mGluR5 are coupled to guanine nucleotide binding Gq proteins and upon activation stimulate phosphoinositide (PI) hydrolysis resulting in an increase in intracellular calcium concentrations (Abe et al. 1992). Splice variants of both mGluR1 (mGluR1a-c; (Pin et al. 1992;Tanabe et al. 1992) and mGluR5 exist (mGluR5a and mGluR5b; (Abe et al. 1992;Joly et al. 1995;Minakami et al. 1993)) and mGluR1a, mGluR5a, and mGluR5b all function to induce PI hydrolysis in a similar manner (Joly et al. 1995). The activation of group I mGluRs (mGluR1 and mGluR5) modulates neuronal excitability and increases intracellular calcium levels, which makes these receptors important modulators of neuronal plasticity.

The mGlu5 receptor is of special interest since these receptors are located within reward-related brain regions. Studies using *in situ* hybridization to detect mRNA levels of subtype-specific mGluRs have demonstrated that expression levels of mGluR5 are high in the rat basal ganglia, including the NAc, caudate/putamen, hippocampus and frontal cortex (Kerner et al. 1997;Lu et al.

1999;Testa et al. 1994a), and these results were confirmed by receptor protein detection (Romano et al. 1995). Deposition of Fluoro-Gold retrograde dye into the VP, a region of moderate mGluR5 expression, reveals that approximately 82% of mGluR5 mRNA containing neurons in the NAc project to this region (Lu et al. 1999). The mGlu5 receptor is located primarily post-synaptically. Indeed, electron microscopy reveals the predominant expression of mGluR5 protein in the cortex, hippocampus and NAc is on dendritic spines, though slight expression of mGluR5 on pre-synaptic axon terminals occurs in these brain regions (Mitrano and Smith 2007;Romano et al. 1995). The localization of mGluR5 on post-synaptic neurons within the limbic brain regions makes them a suitable target for study on stimulant-induced reward.

Subtype-selective mGluR5 allosteric modulator ligands have unique characteristics that gives them potential for novel substance use disorder pharmacotherapy. These ligands do not bind to the orthosteric agonist/antagonist binding site but instead bind non-competitively to a specific site located within the seven transmembrane-domain region of the receptor. Most allosteric modulators are active only when endogenous agonist is bound to the receptor in order to physiologically potentiate or decrease effects of the agonist itself. Through this mechanism of action, the use of allosteric modulators decreases the likelihood of unwanted side effects that occur with traditional agonist/antagonist therapies that are often plagued with compensatory effects subsequent to receptor over/under-activation (see for review (Wang et al. 2009)). The ligand 2-methyl-6-(phenylethynyl)-pyridine

(MPEP) binds to specific residues within the seven-transmembrane spanning region in order to negatively modulate mGluR5 (Malherbe et al. 2003). MPEP is found to specifically decrease agonist-induced activity of mGluR5 and is systemically active (Gasparini et al. 1999). However, MPEP is also shown to have inverse agonist activities in reducing the constitutive activity of mGluR5 (Goudet et al. 2004;Muhlemann et al. 2005;Pagano et al. 2000). The ligand 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) acts at the same allosteric site as MPEP but has greater potency and selectivity for the mGluR5 (Cosford et al. 2003). Both MPEP and MTEP are useful, systemically active, pharmacological tools for determining the functional role of mGluR5 *in vivo*.

The use of allosteric modulators demonstrates the functional importance of mGluR5 in stimulant-mediated behaviors. When MPEP (50mg/kg, i.p.) is given after the acquisition of amphetamine-induced associative learning on the test day, it blunts the expression of Meth-induced CPP in rats (Herzig et al. 2005). Additionally, MPEP (100nmol, intracerebroventricular) given prior to Meth pairing in a specific context hinders the development of the Meth-induced place conditioning in mice (Miyatake et al. 2005). Therefore, the use of MPEP demonstrates the importance of mGluR5 activation during both the development and expression phases of stimulant-induced associative learning processes. Another mGluR5 NAM, MTEP, has been used in operant tasks to demonstrate the reinforcing properties of stimulants. Administration of MTEP dose-dependently (at 1 and 3mg/kg) reduces the self-administration of Meth without altering operant responding for a natural food reinforcer (Osborne and Olive

2008). Furthermore, MTEP (3mg/kg, i.p.) reduces the total number of lever responses in a progressive ratio paradigm of Meth self-administrations, as well as the total number of Meth infusion reinforcers earned (Gass et al. 2009). Moreover, after stable self-administration is acquired and the association between the lever and Meth infusion is extinguished, a pre-treatment of MTEP (1 and 3mg/kg, i.p.) reduces cue and Meth-primed reinstatement of drug-seeking behavior. Throughout these studies, responding for food remains unaltered by MTEP treatments up to the highest dose tested, 3mg/kg (Gass et al. 2009). This collective evidence suggests that mGluR5 activation is needed for the acquisition and retrieval of cue- and Meth-primed drug-seeking behaviors. An area that remains to be explored, however, is the role of mGluR5 in the maintenance of stimulant-associated memories. This is a critical gap for the treatment of substance use disorders and therapeutic intervention at this time period would aid relapse prevention in the drug-withdrawn substance abuser.

The expression and cellular localization of glutamate receptor proteins, including mGluR5, can be altered by stimulant administration. Much of the literature to date reports the consequences of cocaine administration on glutamate receptor protein levels; less is known about the effects of Meth or Amph. Studies utilizing synaptosomal fractionation procedures determined that 21 days after a sensitizing course of cocaine administration there are enhanced expression levels of mGluR5 as well as AMPA and NMDA receptor subunits in the membrane fraction of mPFC (Ghasemzadeh et al. 2009b) and NAc tissue (Ghasemzadeh et al. 2009a). These effects are not observed one day after the end

of cocaine treatment (Ghasemzadeh et al. 2009a;Ghasemzadeh et al. 2009b). The Wolf laboratory also demonstrated an increase the surface expression of AMPA receptor subunits, GluR1 and GluR2/3 at 21 days, and GluR1 and GluR2 in the NAc at 14 days, but not one day, following cocaine-induced sensitization (Boudreau et al. 2007;Boudreau and Wolf 2005). Furthermore, one day after an acute challenge administration of cocaine eliciting expression of motor sensitization, surface expression levels of GluR1 and GluR2 AMPA receptor subunits decreased in the NAc (Boudreau et al. 2007). However, 21 days following a repeated Amph treatment course that induced motor sensitization, GluR1 and GluR2 membrane surface expression levels remain unchanged in the NAc (Nelson et al. 2009). Metabotropic glutamate receptor mRNA levels *are* altered following Amph-induced sensitization in the NAc. Three hours after repeated Amph, mGluR1 levels are increased and mGluR5 levels are decreased in this brain region. While the alterations in mGluR1 are transient, mGluR5 levels remain decreased up to at least 28 days after the last Amph treatment (Mao and Wang 2001). Similar results are reported using synaptosomal fragmentation subsequent to a single, acute injection of Amph in the striatum (Shaffer et al. 2010). That is, one hr after Amph administration, mGluR5 membrane fraction levels are decreased, but the decreases are transient since they normalize by five hrs post-Amph (Shaffer et al. 2010). Shaffer and colleagues also observe a transient increase in mGluR5 levels in the mPFC one hr after Amph that is also normalized by five hr, while no alterations in mGluR1 expression were found in the striatum or mPFC subsequent to Amph treatment (Shaffer et al. 2010). These

collective results demonstrate that glutamate receptor redistribution following cocaine sensitization enhances over the course of time. However, glutamate receptor trafficking is differentially regulated by stimulants cocaine and Amph. Therefore, investigation into the effects of the stimulant Meth on changes in glutamate receptor profiles in reward-related brain regions will fill a gap in our understanding of the literature.

The mGlu5 receptors influence neuronal plasticity through the modulation of ionotoropic glutamate receptors. The activation of group I mGluRs (mGluR1 and mGluR5) in hippocampal neurons gives rise to NMDA and AMPA receptor internalization, which results in a loss of electrophysiological function determined by a decrease in excitatory post synaptic potentials (Snyder et al. 2001). Zhang and colleagues show a mechanism by which mGluR1/5-induced internalization of AMPA receptor subunits occurs. Internalization and de-phosphorylation of GluR1 and GluR2 subunits induced by mGluR1/5 activation was reversed by inhibition of the striatal enriched protein tyrosine phosphatase (STEP) and this effect is specific to mGluR5. Furthermore, activation of mGluR1/5 results in an increase in synaptic fragments of $STEP_{61}$ isoform in hippocampal tissue (Zhang et al. 2008). The $STEP_{61}$ isoform occurs as a result of alternative splicing and is named for its molecular weight of 61kDa (Sharma et al. 1995). This specific STEP isoform is associated with membranes and localized to the endoplasmic reticulum of neurons (Bult et al. 1996). The inhibition of STEP activity in the striatum results in blockade of Amph-induced motor sensitization, which illustrates that the function of STEP is behaviorally relevant to stimulant-

mediated behaviors (Tashev et al. 2009). Specific activation of mGluR5 results in dephosphorylation and subsequent internalization of AMPA receptor subunits *via* $STEP_{61}$ activity which may be a necessary component of Amph-induced motor sensitization.

Significance

The goal of the current dissertation was to expand our knowledge of the role of the mGluR5 system in Meth addiction and in the co-morbidity of schizophrenia and Meth use disorders. First, we sought to determine the role of mGluR5 in the maintenance of Meth-induced associative learning and we further characterized cellular adaptations of mGluR5 in reward-related brain regions at two behaviorally relevant time points. From the literature review, it can be seen that advanced biochemical assays are being utilized to determine the cellular adaptations of the glutamate system including mGluR5 subsequent to cocaine administration. However, the dynamics of mGluR5 following Meth-induced motor sensitization and associative learning remain unknown. Our findings will help guide the field of psychostimulant addiction and determine if mGluR5 could be considered as a potential target for future pharmacotherapies. Our second focus was the effects of Meth exposure in pharmacological and developmental rodent models of schizophrenia. Since the neurocircuitry of stimulant addiction and schizophrenia show clear overlap, we sought to determine how behavioral outcomes of each might be correlated in individual rodents. Rodent models of

schizophrenia demonstrate sensitivity to the effects of stimulant. We reveal here a direct correlation between sensorimotor gating deficits associated with schizophrenia and Meth-induced sensitization and associative learning. We determined that augmenting mGluR5 signaling also increase Meth-induced associative learning in a developmental rodent model of schizophrenia. These novel findings will add to the knowledge of behavioral dysfunction associated with the co-occurrence of Meth abuse and schizophrenia.

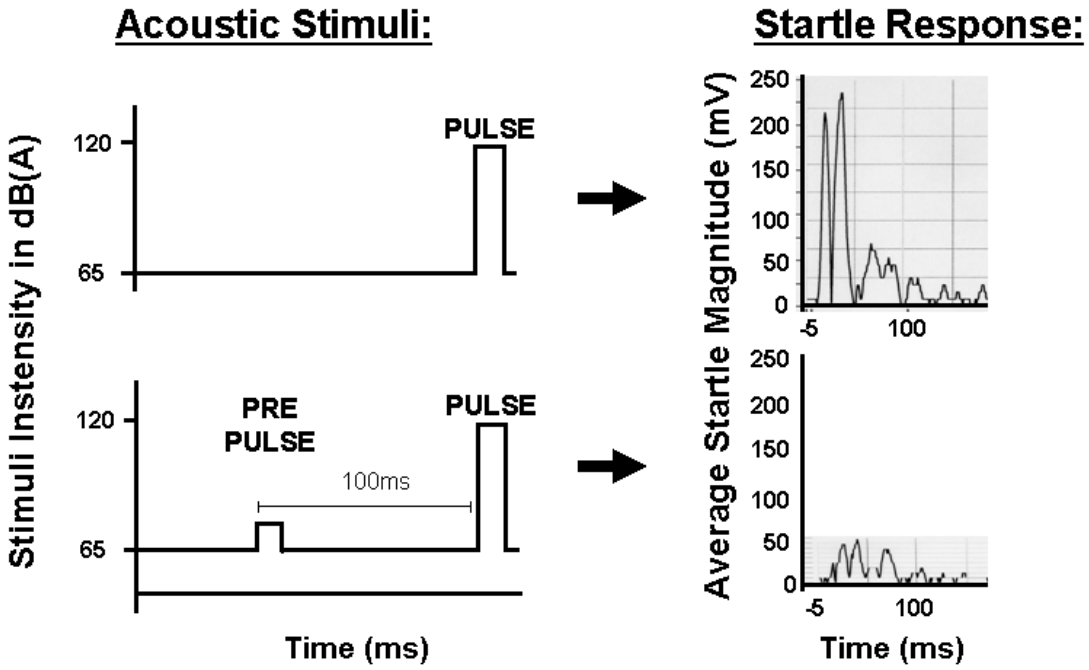


Figure 1. Schematic of the prepulse inhibition (PPI) of the acoustic startle response in rodents. When a strong acoustic stimulus (PULSE) of 120dB(A) is presented alone a large startle response subsequently occurs. When a weaker acoustic stimulus (PRE PULSE) between 68-77dB(A), for example, is presented before the strong, startling PULSE stimulus, the startle response is diminished. The average startle magnitude that is measured is a result of the force the rat exerts on the enclosure in the PPI apparatus that is coupled to a sensor that transmits the information to the PC computer. Figure is modified from Swerdlow & Geyer 1998 *Schizophrenia Bulletin* 24(2):285-301 and includes original data traces of average startle magnitude obtained by A. Herrold in saline treated male Sprague-Dawley rats.

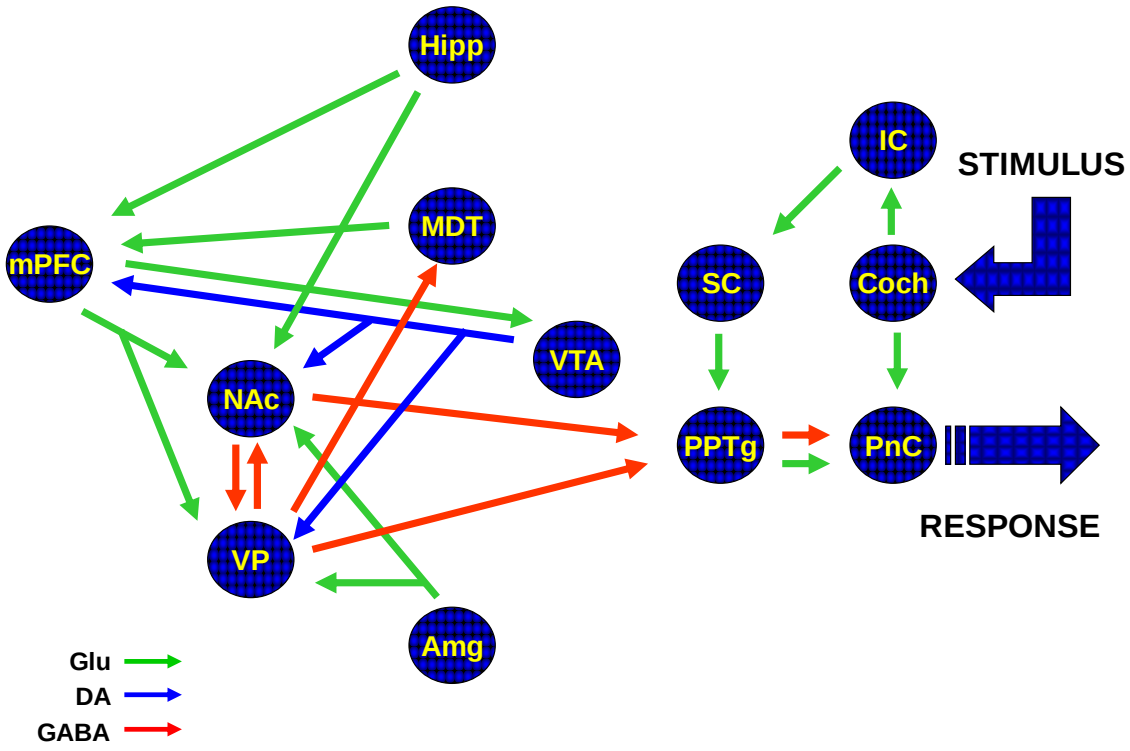


Figure 2. Neurocircuitry of reward and sensorimotor gating. Pictured here are the brain structures which are involved in stimulant-induced behaviors which also modulate the primary circuitry responsible for mediating the acoustic startle response. Glutamate (Glu) projections are depicted in green, dopaminergic (DA) in blue and GABAergic (GABA) in red. mPFC, medial prefrontal cortex; NAc, nucleus accumbens; VP, ventral pallidum; Hipp, hippocampus; MDT, medial dorsal thalamus; Amg, amygdala; VTA, ventral tegmental area; PPTg, pedunculopontine nucleus; PnC, nucleus reticularis pontis caudalis; SC, superior colliculus, IC, inferior colliculus; Coch, cochlear nucleus.

CHAPTER III

RATIONALE FOR METHODOLOGY AND PRELIMINARY STUDIES

Methamphetamine conditioned place preference dose determination

To determine the dose of Meth to be used with repeated conditioning sessions in the CPP task to induce a reliable and persistent preference of the Meth-paired context, a Meth dose-response study (0.1, 0.3, and 1.0mg/kg, in the volume of 1ml/kg (i.p.)) was conducted using 36 male Sprague-Dawley rats. The CPP experiment consisted of three phases during which activity and time spent in each compartment was monitored: pre-test, conditioning, and CPP tests. For the pre-test, rats were given access to the entire CPP box (see Fig. 3B) in a drug-free state for 30min. The CPP box consists of two large chambers with distinct yet neutral cues separated by sliding Plexiglas doors from a smaller center chamber with a smooth white floor and white sides (Fig.3C&D). Rats were then assigned to Meth and saline paired chambers in a counter-balanced manner. That is, half of the rats were paired with Meth in the chamber of the CPP box where they spent the least amount of time on the pre-test and the other half were paired with saline in the chamber where they spent the greatest amount of time on the pre-test. Also, the time spent in each of the large chambers during the pre-test was balanced so that there was no statistical difference between the sides for which they were paired for each group.

Rats were then administered Meth at a dose of 0.1, 0.3, or 1.0mg/kg, i.p. on days 1, 3, and 5 and then immediately placed in the assigned, Meth-paired chamber for 45min. On days 2, 4, and 6, rats were administered saline (1ml/kg, i.p.) and then immediately placed in the opposite compartment for 45min. Three days later, rats were tested for conditioned preference (CPP Test 1) in a drug-free state. Then rats were repeatedly tested for persistence of preference weekly up to CPP Test 3 (see Fig. 4A for experimental timeline). A two-way repeated measures ANOVA statistical analysis with a *post hoc* Newman Keuls test for multiple comparisons was conducted to determine preference; preference was defined as a significantly greater amount of time being spent in the Meth-paired compared to the saline-paired chamber. Rats that spent greater than two standard deviations above or below the mean in any chamber on any test day were removed from statistical analysis. Rats conditioned with Meth at a dose of 0.1mg/kg (n=10) demonstrated a significant preference for the Meth-paired chamber on CPP Tests 1 and 2 (*post hoc* Newman Keuls, $p<0.01$; effect of Chamber $F_{(1,18)}=27.917$, $p<0.0001$; effect of CPP Test Day $F_{(2,36)}=0.008$, $p=0.992$; Interaction $F_{(2,36)}=3.670$, $p=0.0035$, Fig.4B). Rats conditioned with Meth at a dose of 0.3mg/kg (n=10) significantly preferred the Meth-paired chamber over the saline-paired chamber on CPP Tests 1 and 2 (*post hoc* Newman Keuls, $p<0.01$; effect of Chamber $F_{(1,18)}=10.542$, $p=0.004$; effect of CPP Test Day $F_{(2,36)}=0.034$, $p=0.967$; Interaction $F_{(2,36)}=10.998$, $p=0.002$, Fig. 4C). Rats conditioned with Meth at a dose of 1.0mg/kg (n=10) demonstrated a significant preference for the Meth- compared to the saline-paired chamber on CPP Tests 1, 2, and 3 (*post hoc*

Newman Keuls, $p<0.01$; effect of Chamber $F_{(1,18)}=31.451$, $p<0.0001$; effect of CPP Test Day $F_{(2,36)}=0.023$, $p=0.977$; Interaction $F_{(2,36)}=2.271$, $p=0.118$, Fig. 4D). Therefore, the 1.0mg/kg Meth dose appeared to provide the most consistent and enduring preference in this conditioning paradigm, thus it was chosen for the completion of the current dissertation work. For future studies that will determine antagonism (Chapter IV) and correlation of CPP with other behavioral outcomes (Chapter VII), a different experimental design was used in which the rats are paired with Meth on the side where they spent the least amount of time on the pre-test. Pairing rats with a drug on the side in which they spent the least amount of time on the pre-test raises some concern as to whether reward or anxiolytic properties of the drug are being assessed. However, studies in our lab were conducted in order to determine the anxiolytic properties of the Meth dosing regimen chosen using an elevated plus maze. Work completed by Robin Voigt in the Napier lab has determined that neither acute nor repeated treatment of Meth (1mg/kg, i.p.) produced anxiolytic or anxiogenic properties compared to saline (1ml/kg, i.p.) treatment. Studies in the published literature have employed the method of pairing rodents with a rewarding stimulant on the side in which the least amount of time was spent in the pre-test during conditioning (Li et al. 2001;Nomikos and Spyraki 1988). Thus, we felt comfortable moving forward with this behavioral treatment paradigm with Meth CPP.

Motor activity was also assessed at each conditioning session (after the initial 10min where animals are settling down from initial injection). Rats were removed from motor assessments for an individual activity parameter if activity

counts for Day 1 or Day 5 were greater than two standard deviations above or below the mean. A two-way repeated measures ANOVA with *post hoc* Newman Keuls test for multiple comparisons was utilized to determine the effects of each dose of Meth on motor activity on the first (Day 1) and last (Day 5) day of administration and to compare acute effects of each dose. Rats administered Meth at a dose of 1.0mg/kg demonstrated increased horizontal activity (Fig. 5A) and time spent in stereotypy (i.e., repetitive movements; Fig. 5C) on conditioning Day 1 compared to rats administered 0.3 or 0.1mg/kg Meth (two-way repeated measures ANOVA with *post hoc* Newman Keuls test, $p<0.01$). However, Meth administration in this paradigm at any dose tested failed to develop motor sensitization (i.e., there was not an increase in motor activity on Day 1 compared to Day 5 for any motor parameter at any Meth dose tested). Statistical results for each motor parameter: Horizontal activity; Dose $F_{(2,31)}=27.134$, $p<0.0001$, Test Day $F_{(1,31)}=1.846$, $p=0.184$, Interaction $F_{(2,31)}=4.144$, $p=0.025$. Vertical activity; Dose $F_{(2,30)}=14.278$, $p<0.0001$, Test Day $F_{(1,30)}=0.0005$, $p=0.983$, Interaction $F_{(2,30)}=3.793$, $p=0.034$. Stereotypy time; Dose $F_{(2,31)}=21.424$, $p<0.0001$, Test Day $F_{(1,31)}=2.480$, $p=0.125$, Interaction $F_{(2,31)}=2.874$, $p=0.072$. We have found in the Napier laboratory that while this treatment course of Meth reliably induces CPP, motor sensitization does not always occur. This could be due to the Meth dose or treatment interval, since Meth administered daily for five days at a dose of 2.5mg/kg (s.c.) has induced motor sensitization in the Napier laboratory. These data also demonstrate that CPP and motor sensitization can be dissociated and likely model different aspects of the addiction phenomenon.

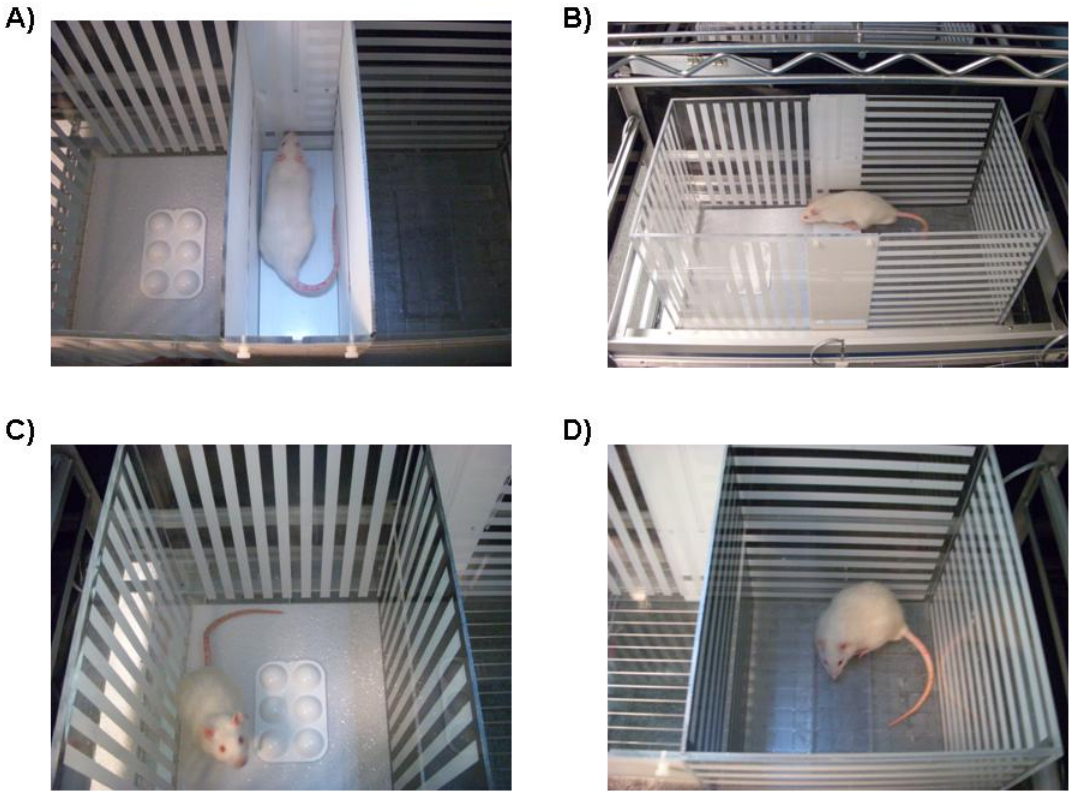


Figure 3. Behavioral testing apparatus. A total of 16 test boxes were used for the completion of the current dissertation acquired from Accuscan Instruments, Inc., Columbus, OH. **A)** The CPP box consists of two larger chambers (25cm x 30cm x 30cm) separated with sliding Plexiglas doors by a smaller center chamber (13cm x 30cm x 30cm) equipped with two banks of photobeams attached to the metal frame (24 horizontal and 12 vertical photobeams). At the start of the pre-test or CPP test, the doors placed inside the box. The rat is then placed in the small center chamber enclosed by the sliding doors. **B)** Immediately after the rat is placed in the center chamber, the sliding

doors are removed to allow the rat free access to the entire CPP box during the pre-test or CPP test for 30min. **C)** During conditioning sessions, rats are placed in an assigned chamber separated by one sliding door for 45min. Each larger chamber has distinct, yet neutral visual and tactile cues. Pictured here is the white, opaque patterned floor with an over-turned paint dish glued to the center with epoxy. The visual cues are the vertical stripes on the surrounding walls of the chamber. **D)** Pictured here is an alternative chamber configuration with the grid floor and Plexiglas rectangular insert glued with epoxy to the center of the floor. The visual cues are horizontal stripes on the surrounding walls of the chamber. The floors are removable and floor type is randomized with visual cues in the CPP box during the assignment of chamber for conditioning.

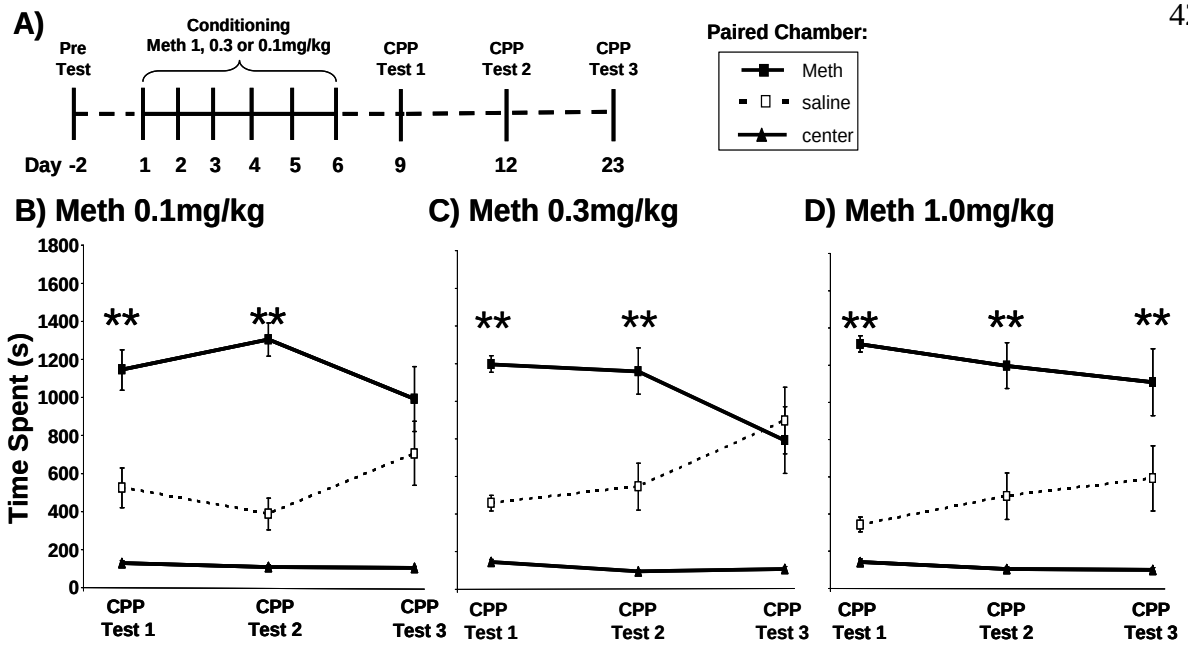


Figure 4. Methamphetamine dose response CPP study. A) Timeline for behavioral experimentation. Rats were pre-tested in a drug free state on day -2. Three days later, rats were given Meth at a dose of 0.1mg/kg (N=10), 0.3mg/kg (N=10), or 1.0mg/kg (N=10) on days 1, 3, and 5 and placed in their assigned chamber. All rats were administered saline (1ml/kg, i.p.) and placed in the opposite chamber on days 2, 4, and 6. Rats were then tested for initial preference on day 9 (CPP Test 1) in a drug-free state. Rats were then repeatedly tested for persistence of preference in a drug-free state on day 12 (CPP Test 2) and day 23 (CPP Test 3). Solid lines with filled squares represent time spent on the Meth-paired chamber. Dotted lines with empty squares represent time spent on the saline-paired chamber. Solid lines with filled triangles represent time spent in the center chamber (center chamber not included in statistical measures, only used for visual comparison). **B)** Rats conditioned with Meth at a dose of 0.1mg/kg (i.p.) only expressed a significant preference for the Meth-paired

chamber on CPP Tests 1 and 2. **C)** Rats conditioned with Meth at a dose of 0.3m/kg (i.p.) spent significantly more time in the Meth-paired chamber on CPP Tests 1 and 2. **D)** Rats conditioned with Meth at a dose of 1.0mg/kg (i.p.) expressed a significant preference for the Meth-paired chamber on CPP Tests 1 to 3. Two-way repeated measures ANOVA with *post-hoc* Newman Keuls, **p<0.01.

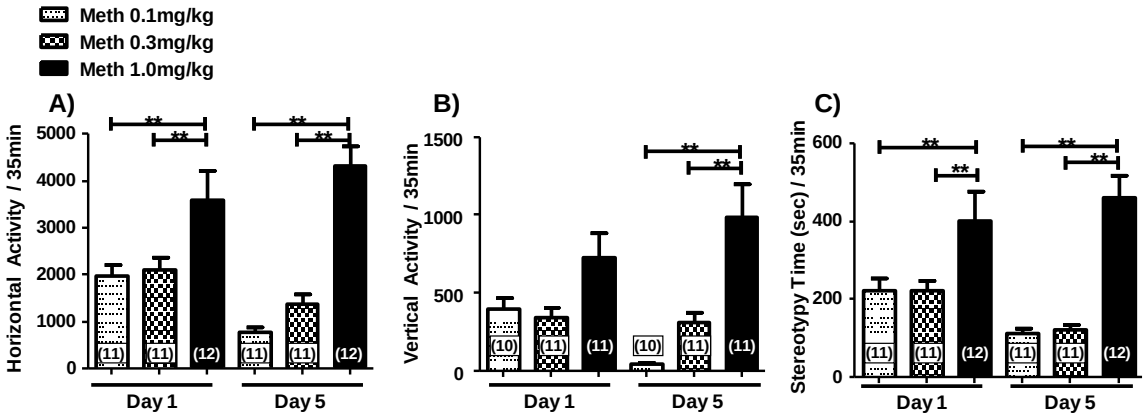


Figure 5. Motor activity induced by Meth during conditioning. Motor activity was assessed on conditioning Day 1 and Day 5 (Fig. 4A) for all doses of Meth (0.1, 0.3, and 1.0mg/kg, see key) for the last 35min of the conditioning session. Sample size is represented within bars (n). **A)** Rats treated with 1.0mg/kg Meth demonstrated increased horizontal activity on Day 1 and Day 5 compared to rats treated with 0.3 or 0.1mg/kg Meth during conditioning. However, rats treated with 0.1, 0.3 or 1.0mg/kg Meth did not demonstrate an increase in horizontal activity on Day 5 compared to Day 1. **B)** There was an increase in vertical activity in rats treated with 1mg/kg Meth compared to rats treated with 0.1 or 0.3mg/kg on Day 5. **C)** Rats treated with 1.0mg/kg Meth demonstrated increased stereotypy time on Day 1 and Day 5 compared to rats treated with 0.3 or 0.1mg/kg Meth during conditioning. However, there was no difference in stereotypy time between Days 1 and 5 for any Meth dose tested. Two-way repeated measures ANOVA with *post hoc* Newman Keuls test for multiple comparisons, **p<0.01.

mGluR 1/5 allosteric modulator selection

MPEP, 2-methyl-6-(phenyl-ethynyl)-pyridine, mGluR5 NAM

The mGluR5 NAM, MPEP, is systemically active and is mGluR subtype selective (Gasparini et al. 1999). However, this ligand does display some off-target activity at the ionotropic NMDA receptor at high concentrations (NMDAR IC_{50}=18μM; Table 1) (Cosford et al. 2003). The use of MPEP at a dose of 30mg/kg, i.p. has been shown to blunt the development and expression of morphine-induced CPP (Herzig and Schmidt 2004), which has been validated in our lab (Herrold et al. 2005). This dose of MPEP does not produce rewarding effects on its own as assessed in a CPP paradigm (McGeehan and Olive 2003). However, preliminary data from our lab suggests that it blunts motor activity. Because administration of MPEP at a dose of 30mg/kg, i.p. did not blunt the preference for cocaine, and a dose of 50mg/kg, i.p. did not affect preference for ecstasy using CPP, we felt that 30mg/kg, i.p. of MPEP should not disrupt the ability of rats to perform the CPP task. We also determined in preliminary studies that MPEP (30mg/kg, i.p.) given after Meth conditioning but prior to the CPP test (Fig. 6A) failed to disrupt the expression of Meth-induced CPP. These results demonstrated that MPEP at 30mg/kg, i.p. failed to disrupt the maintenance of the Meth-associated context (Fig. 6B&C). These results could be due to the time period at which the mGluR5 NAM was administered, the number

of administrations, or the dose given. In Chapter IV the variable of time period at which mGluR5 is critical for the maintenance of CPP is further addressed.

MTEP, 3-[(2-methyl-1,3-thiazol-4-ul)ethynyl]pyridine, mGluR5 NAM

The novel mGluR5 NAM, MTEP, demonstrates increased selectivity and potency over MPEP (Cosford et al. 2003) (Table 1.); thus, studies included in Chapter IV and VI of the current dissertation project were carried out with this ligand. *In vitro* selectivity data for MTEP demonstrate IC_{50} (half maximal inhibitory concentration) greater than 100μM for the group I subtype 1 metabotropic glutamate receptor (mGluR1) and greater than 300μM over the ionotropic NMDA receptor subunit NR2B (Cosford et al. 2003). Furthermore, full receptor occupancy occurs after a 10mg/kg, i.p., dose of MTEP in rat hippocampal brain tissue (Busse et al. 2004).

The dose of MTEP selected to test the effects of mGluR5 blockade on the maintenance of Meth-induced CPP (Chapter IV) was 3mg/kg, i.p. This dose is the maximal effective dose shown to reduce self-administration of Meth without affecting self administration of food reward (Gass et al. 2009;Osborne and Olive 2008). Furthermore, MTEP at 3mg/kg, i.p. reduced cue- and Meth-induced reinstatement of Meth self administration behavior (Gass et al. 2009). In order to determine if blockade of mGluR5 could block the development of Meth-induced motor sensitization (Chapter VI), a 5mg/kg, i.p., dose of MTEP was used. This dose of MTEP (5mg/kg, i.p.) was chosen since it reduces the expression of cocaine-induced rearing activity, a motor parameter more frequently seen to be

augmented by repeated Meth treatment in our laboratory, without affecting vertical activity in saline pre-treated rats (Dravolina et al. 2006).

JNJ16259685, (3,4-dihydro-2H-pyranol[2,3]b quinolin-7-yl)(cis-4-methoxycylohexyl)methanone, mGluR1 NAM

The mGluR1 NAM, JNJ16259685, is selective, systemically active, and highly potent drug (Table 1). *In vivo* potency data reveal an ED_{50} (half maximal effective dose) equal to 0.04mg/kg, s.c. in the rat cerebellum and 0.014mg/kg, s.c (Lavreysen et al. 2004). Furthermore, *in vitro* data demonstrate JNJ16259685 to be a selective ligand without non-specific effects on other mGluRs up to concentrations of 10μM (Lavreysen et al. 2004). A dose of 0.3mg/kg JNJ16259685 was used for the current dissertation project (Chapter IV) since it reduces self-administration of ethanol in alcohol preferring rats without effecting responding for sucrose reward (Besheer et al. 2008).

CDPPB, 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide, mGluR5 PAM

The mGluR5 positive allosteric modulator, PAM, CDPPB, is shown to be systemically active and selective. *In vitro* selectivity data for CDPPB demonstrate an IC_{50} greater than 9.7μM for other metabotropic glutamate receptors (mGluR17-4,8). *In vivo* potency data demonstrate an EC_{50} (half maximal effective concentration) of 20nM (Lindsley et al. 2004) (Table 1).

In order to determine if augmenting mGluR5 could enhance Meth-induced place preference in isolation-reared rats (Chapter VII), a 3mg/kg, s.c., dose of

CDPPB was used. This dose of CDPBB (3mg/kg, s.c.) was chosen since it was most effective in facilitating the extinction of cocaine-induced CPP (Gass and Olive 2009). Doses of CDPPB that could be rewarding on their own were also avoided, since this mGluR5 PAM was to be given prior to Meth conditioning in the CPP paradigm. The 3mg/kg CDPPB dose had no effect on Amph-induced motor activity (Kinney et al. 2005). Furthermore, a higher dose of CDPPB (10mg/kg, s.c.) had no effect on extracellular dopamine concentrations in the NAc or mPFC (Lecourtier et al. 2007). Therefore, we felt that a dose of 3mg/kg, s.c. of CDPPB would selectively enhance signaling through mGluR5.

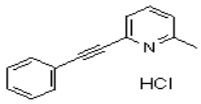
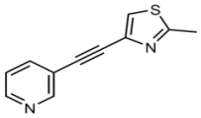
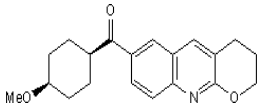
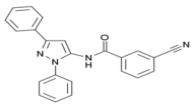
mGluR Compound	Chemical Name	Selectivity	Potency (rat receptor)
MPEP: mGluR5 NAM 	2-methyl-6-(pheylethynyl) pyridine	mGluR1 IC_{50}>100µM NR2B IC_{50}=18µM	ED_{50}=2.1mg/kg, i.p. Hippocampal brain concentration after 3mg/kg = 0.83±0.05µM
MTEP: mGluR5 NAM 	3-([2-methyl-1,3-thiazol-4-ul)ethynyl]pyridine	mGluR1 IC_{50}>100µM NR2B IC_{50}>300µM	ED_{50}=1mg/kg, i.p. Hippocampal brain concentration after 3mg/kg = 1.4±0.2µM
JNJ16259685: mGluR1 NAM 	(3,4-dihydro-2H-pyrano[2,3]b quinolin-7-yl)(*cis*-4-methoxycylohexyl) methanone	mGluR5 IC_{50}=1.31±0.39µM, no activity on mGluR3-4,6 up to 10µM	ED_{50}=0.014mg/kg, s.c. (thalamus) ED_{50}=0.040mg/kg, s.c. (cerebellum)
CDPPB: mGluR5 PAM 	3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide	mGluR1-4, 8 IC_{50}=9.7µM	EC_{50}=20nM

Table 1. mGluR ligands used for the current dissertation project. All mGluR ligands used are group and sub-type selective. Please refer to text for citations.

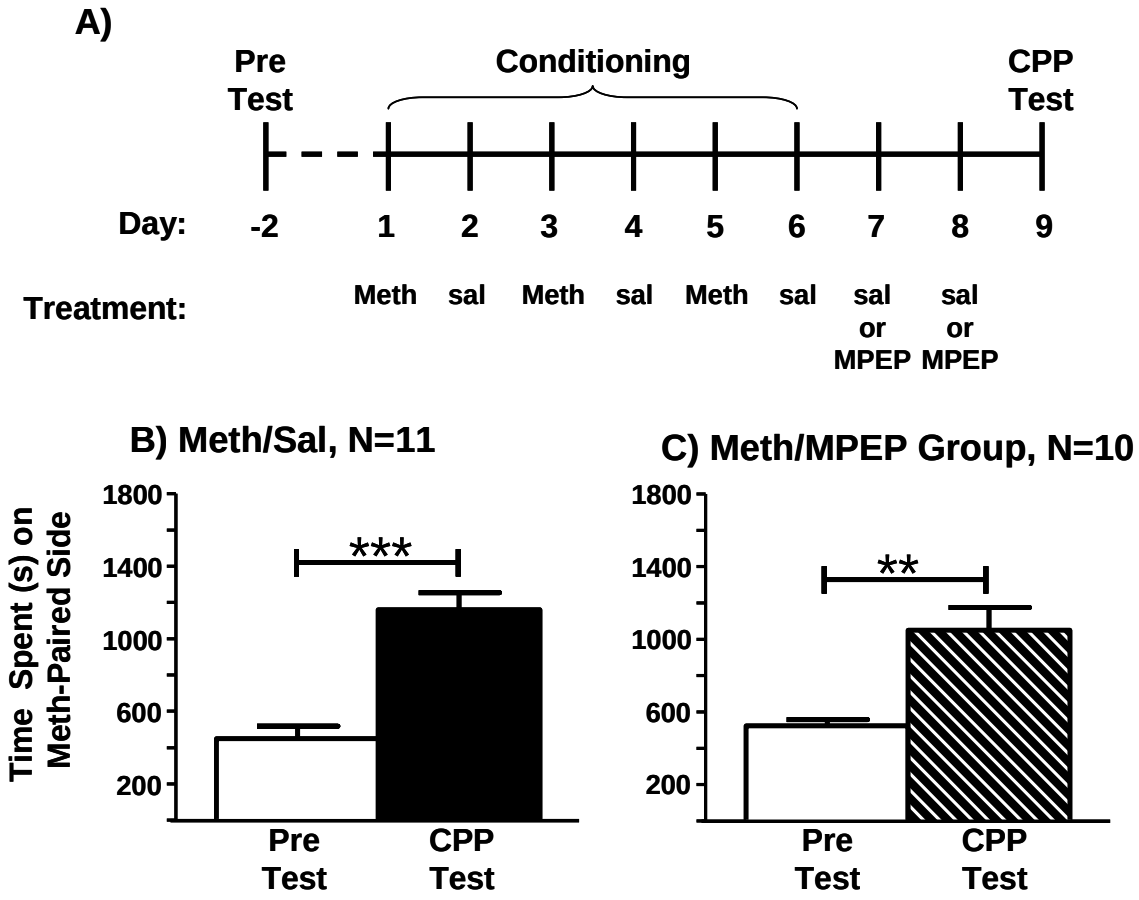


Figure 6. Effects of MPEP (30mg/kg) on maintenance of Meth-induced CPP. A) Timeline of behavioral experiment. Rats were given access to the entire test box on the pre-test (protocol day -2). All rats were then administered Meth (1mg/kg, i.p.) and then placed in the chamber where they spent the least amount of time on the pre-test on days 1, 3, and 5. On days 2, 4, and 6 rats were injected with saline (sal, 1ml/kg, i.p.) and placed in the opposite chamber. Rats were given injections of saline vehicle (3ml/kg, i.p.) or MPEP (30mg/kg, i.p.) in their home cage on days 7 and 8. Conditioning sessions lasted for 45min. Twenty-four hours later, rats were given access to the entire CPP box in a drug-free state on day 9 for 30min. A total of 24 rats were used for the

following comparisons. Eight rats from the Meth/Sal group (conditioned with Meth and given saline on days 7 and 8) were rats used in the Meth dose response study that were paired on the side in which they spent the least amount of time on the pre-test. The remaining 16 rats were tested in a separate behavioral run where 4 were added to the Meth/Sal group, and 12 were administered Meth during conditioning and MPEP on days 7 and 8 (Meth/MPEP group). Rats were removed as outliers if time spent in any chamber on the pre-test or CPP test was greater than two standard deviations above or below the mean. Both the **B)** Meth/Sal group ($t_{(10)}$=5.237, p=0.0004) and the **C)** Meth/MPEP group ($t_{(9)}$=3.846, p=0.004) expressed a significant preference for the Meth-paired chamber after conditioning (CPP Test) compared to the initial preference (Pre Test). Paired *t*-test, **p<0.01, ***p<0.001.

Selection of Rodent Schizophrenia Models

In order to investigate the converging neuropathologies of schizophrenia and stimulant use disorders, we chose to use pharmacological and developmental models of schizophrenia that both exhibit deficits in sensorimotor gating of the acoustic startle response measured by PPI. Deficits in PPI are a well-established behavioral outcome that occurs in schizophrenia patients (Braff et al. 2001) and are easily assessed with high construct validity. Deficits in PPI are induced by Amph administration in both humans and rodents (Swerdlow et al. 2003). Recently, two separate laboratories have published repeated, escalating treatment courses of Amph administration that produce robust deficits in PPI that chronically persist (Peleg-Raibstein et al. 2008;Tenn et al. 2005). This was an important outcome measure, as behavioral assessments of reward were designed to follow and emulate a co-morbid schizophrenia and stimulant addiction brain state. The study conducted by Tenn and colleagues demonstrates that PCP does not produce persistent PPI deficits, which ruled out its use in our behavioral paradigm (Tenn et al. 2005). The Amph treatment course employed by Peleg-Raibstein was utilized in Chapter VII since it allowed for rapid induction (six days as opposed to three weeks as in the Tenn *et al.* 2005 study) of an enduring brain state that is biochemically similar to that of the schizophrenia neuropathology as described in the Literature Review (Chapter II)(Peleg-Raibstein et al. 2008). However, because the use of two such similar stimulants to model separate behavioral outcomes presents itself as a potential confound, we chose to validate our results obtained from the repeated Amph treatment

paradigm with the use of a developmental rodent model of schizophrenia. The early stressor of maternal separation and isolation rearing following weaning is another means of inducing PPI deficits in rodents (Geyer et al. 1993). Unlike the neonatal ventral hippocampal lesion model, isolation rearing is easily executed and does not employ additional surgical techniques. In summary, repeated escalating Amph and isolation rearing are two behavioral models of schizophrenia with high construct validity that allow for sensorimotor gating assessments in a chronic treatment paradigm.

CHAPTER IV

mGluR5 BUT NOT mGluR1 IS NECESSARY FOR MAINTENANCE OF METHAMPHETAMINE-INDUCED ASSOCIATIVE LEARNING

Abstract

Conditioned place preference (CPP) reflects the significance of contextual cues that are repeatedly associated with rewarding effects of abused drugs like methamphetamine (Meth). Glutamate neurotransmission is augmented in response to exposure to stimulants and cues associated with their use. Activation of group I metabotropic glutamate receptors (mGluR) is critical for the acquisition and expression of behavioral tasks mediated by stimulants. We hypothesized that the maintenance of Meth-induced behaviors would also require activated mGluR, and that the role of mGluR1 *versus* mGluR5 may differ. Negative allosteric modulators (NAMs) of these receptors were evaluated, since this class of drugs have the advantage of acting only on agonist- (i.e., glutamate) occupied receptors and thus provide a more targeted action. Conditioning with Meth every other day for six days resulted in significant preference for the Meth-paired compartment.

Two daily injections of the mGluR1 NAM, JNJ16259685 (0.3mg/kg, i.p.) or its vehicle on days 13 and 14 after Meth-conditioning did not influence the maintenance of Meth-induced CPP; however, administration of the mGluR5 NAMs MTEP (3mg/kg, i.p.) and MPEP (30mg/kg, i.p.) inhibited maintenance processes necessary for CPP to be expressed. These findings demonstrate a subtype-specific role of mGluR5 receptors in the maintenance of place preference memory and potential of mGluR5 NAMs as a useful target for addiction therapy.

Introduction

Methamphetamine (Meth) addiction is a problem of global health concern for which there is no FDA-approved pharmacotherapy (Elkashef et al. 2008). Even after protracted abstinence, Meth-addicted individuals are prone to cue-elicited relapse (Hartz et al. 2001). One aspect of addiction that makes it such a persistent phenomenon is the strength of learned associations between the rewarding effects of drugs and the context in which drugs are administered (O'Brien et al. 1992). Drug-induced associative learning can be studied in rodents and humans using conditioned place preference (CPP) (Childs and deWit H. 2009;Tzschentke 2007).

Withdrawal from stimulant treatment results in dynamic changes within the cortico-striatal glutamate transmitter system. At early time points after stimulant administration, small transient changes in glutamate receptor proteins occur, perhaps as a compensatory response to drug-induced glutamate transmitter levels in brain regions such as the nucleus accumbens (NAc) and

medial prefrontal cortex (mPFC) (Ghasemzadeh et al. 2009a;Ghasemzadeh et al. 2009b;Shaffer et al. 2010;Shoblock et al. 2003). During extended withdrawal from long-term cocaine abuse, the frontal cortex of humans is hypoactive (Bolla et al. 2004;Goldstein and Volkow 2002). In rats withdrawal from repeated stimulant exposure reduces glutamate levels in NAc and mPFC (Baker et al. 2003;Lominac and Szumlinski 2008), and ionotropic and metabotropic glutamate receptors are increased in these brain regions (Ary and Szumlinski 2007;Boudreau and Wolf 2005;Ghasemzadeh et al. 2009a;Ghasemzadeh et al. 2009b). After an extended withdrawal in human stimulant abusers, the FC becomes hyper-responsive to drug-associated cues (Childress et al. 1999;Grant et al. 1996). In stimulant-withdrawn rodents, re-exposure to drug or drug-associated cues increases limbic glutamate levels beyond that seen in rats without a stimulant treatment history (Chen et al. 2001;Pierce et al. 1996;Qi et al. 2009). Drug cues hinder abstinence in addiction; therefore, there should be great therapeutic value in normalizing the glutamatergic responses to drug-associated cues.

Glutamate acts on both ionotropic and metabotropic receptors. Group I mGluRs include both metabotropic glutamate receptor subtype 1 (mGluR1) and 5 (mGluR5), which are located primarily post-synaptically (Conn and Pin 1997). Because of the localization of group I mGluRs within the brain systems important for stimulant-mediated behavior, negative allosteric modulators (NAMs) of mGluR1 and 5 may provide an important avenue to normalize the hyper-responsive glutamate system after repeated stimulant administration (Lu et al.

1999;Romano et al. 1995;Testa et al. 1994a). Binding to sites that are remote from the orthostatic position, allosteric modulators alter the efficacy of endogenous agonists (Pin et al. 2003;Wang et al. 2009). Allosteric modulators also avoid many of the unwanted side effects that can occur with direct acting agonists/antagonists, where compensatory effects and widespread action are prevalent (Wang et al. 2009). Group I mGluR NAMs have recently been used in rodent models of addiction (Carroll 2008;Olive 2009). The mGluR5 NAM 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) decreases self-administration of Meth, as well as cue- and Meth-primed reinstatement of Meth self-administration (Gass et al. 2009;Osborne and Olive 2008). Likewise, the related NAM, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), reduces the development and expression of Meth- and amphetamine-induced associative learning, respectively (Herzig et al. 2005;Miyatake et al. 2005). The mGluR1 NAM, JNJ16259685, decreases ethanol self-administration (Besheer et al. 2008), and another mGluR1 NAM, EMQMCM, reduces cocaine-induced motor activity (Dravolina et al. 2006) as well as cue- and drug-induced reinstatement of nicotine self administration in rodents (Dravolina et al. 2007). While negative modulation of mGluR1 and mGluR5 decreases reward-mediated behaviors, evidence suggests that these receptor subtypes may play different roles in acquisition, consolidation and retention of memory (Salinska 2006;Simonyi et al. 2007;Steckler et al. 2005). For example, the mGluR1 receptor is important for the acquisition, while the mGluR5 receptor may be more critical for the retention, of spatial memories (Steckler et al. 2005). What remains unknown is the role of

these receptors, and potential subtype-specific effects, in the maintenance of stimulant-associated memories.

The maintenance of drug memories underlies relapse; therefore, a better understanding of the role of group I mGluRs in this relationship may aid relapse reduction. We hypothesize that post-conditioning administration of mGluR5 NAMs will disrupt the Meth – context memory association and thus decrease the subsequent expression of Meth-induced CPP. We chose to administer therapeutic interventions at 13 and 14 days post-conditioning, a time frame when extracellular limbic glutamate is increased (Chen et al. 2001), and our lab has shown neuronal changes subsequent to repeated Meth administration (McDaid et al. 2006b), and when there is evidence of persistent up-regulation in glutamate receptors (Ghasemzadeh et al. 2009a;Ghasemzadeh et al. 2009b). Comparison of the mGluR1 and mGluR5 NAMs will allow us to differentiate between the involvements of the mGluR subtypes in this Meth-mediated behavior.

Materials and Methods

Animals

Eighty male, 225-250g, Sprague-Dawley rats were used. Rats were treated in accordance with NIH Guide for the care and use of laboratory animals and the Rush University Institutional Animal Care and Use Committee. Rats were allowed to habituate to the environmentally controlled *vivarium* (23-25°C; 7:00AM/7:00PM light dark cycle) at least one week prior to the start of

behavioral testing and given food and water *ad libitum*. All studies were conducted during the light phase.

Drugs

(+)Methamphetamine hydrochloride (Meth, Sigma-Aldrich, St. Louis, MO) in 0.9% NaCl sterile saline was given at a dose of 1mg/kg as the base. Our lab and others have used this dose to successfully induce Meth CPP in rats (Herrold et al. 2009;Kitanaka et al. 2010;Li et al. 2002;Schindler et al. 2002). The mGluR1 NAM, JNJ16259685 (Tocris Bioscience, Ellisville, MO) was administered at a dose of 0.3mg/kg, which reduces ethanol self-administration without reducing natural rewards (Besheer et al. 2008). The mGluR5 NAM, 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP, Tocris Bioscience) was administered at a dose of 3mg/kg, which reduces Meth self-administration (Osborne and Olive 2008) as well as cue- and drug-induced reinstatement of Meth self-administration (Gass et al. 2009). The mGluR5 NAM 2-methyl-6-(phenyl-ethynyl)-pyridine (MPEP; a gift from Novartis Institutes for BioMedical Research; Basel, Switzerland) was administered at a dose of 30mg/kg, which reduces the development and expression of morphine-induced CPP (Herzig and Schmidt 2004). All NAMs were dissolved in 10% w/v (2-hydroxypropyl)-γ-cyclodextrin in sterile water solution (termed vehicle, Sigma-Aldrich). All drugs and their vehicles were given *via* intraperitoneal (i.p.) injection in a volume of 1ml/kg.

Conditioned Place Preference (CPP)

CPP boxes (Accuscan Instruments, Inc., Columbus, OH) used for the study are described in a previous publication from our laboratory (Shen et al. 2006). Rats were transported to the behavioral testing room at least 30min prior to experimentation. Timeline of the behavioral paradigm is illustrated in Fig. 7A. All rats were given a pretest that demonstrated no significant group preferences for either chamber. Some individual rats tended to prefer one chamber over the other; thus, rats were paired with Meth in the chamber in which they spent the least amount of time during the pretest. Rats were treated with Meth on days 1, 3 and 5, and saline on days 2, 4 and 6 and then immediately placed in the appropriate chamber for 45min. On day 9, untreated rats were tested for chamber preference (CPP Test 1) by giving them free access to the entire box for 30min. Following the procedures employed by Paolone and colleagues (Paolone et al. 2009), if the difference between pretest and CPP Test 1 was at least a 10% (>180s), the rats were used for assessing the effects NAMs. This helped assure a sufficient 'signal' was used to ascertain the antagonism capacity of mGluR1/5 NAMs (54 out of 80 rats met this criterion). On days 10-17, all rats were given once daily vehicle (1ml/kg) injections in their home cage. On days 18 and 19, rats were given in their home cage a once-daily injection of either vehicle (1ml/kg), JNJ16259685 (0.3mg/kg), MTEP (3mg/kg), or MPEP (30mg/kg). On day 22, rats were tested for preference and motor activity was assessed (CPP Test 2) as described previously for CPP Test 1 in a drug-free state.

Statistics

Differences in time spent per chamber between pretest and CPP Test 1 was determined *via* a paired *t*-test. Preference was determined based on a significant increase in amount of time spent on the Meth- *vs*. saline-paired chamber on CPP Test 1 and 2 *via* a two- way repeated measure (rm) ANOVA with *post hoc* Newman Keuls test with $\alpha=0.05$ for all tests. Statistical outliers for a given measure were removed if data fell outside 2 standard deviations from the mean. Data are represented as the mean $\pm$ standard error of the mean (SEM).

Results

The time spent in the two large chambers was not different during the pretest for all rats tested (846.3±42.9sec *vs*. 813.1±42.6sec; $t_{(79)}=0.391$, p=0.697, N=80). Three days after conditioning, rats demonstrated a robust preference for the Meth- (930.3±32.0sec) *vs*. the saline-paired chamber (694.1±31.6sec, $t_{(79)}=3.732$, p<0.001, n=80) as a whole. Rats that showed more than a 10% change in preference on the pretest *vs*. CPP Test 1 (n=47), spent 1097.7±28.4sec in the Meth-paired chamber and 539.7±28.2sec in the saline-paired chamber during CPP Test 1 ($t_{(46)}=9.913$, p<0.001). Thus, the culling procedure does not drastically alter behavioral outcomes.

Rats given post-conditioning vehicle (Fig. 7B, n=11) or JNJ16259685 (Fig. 7C, n=12) spent significantly more time in the Meth- *vs*. saline-paired chamber on both CPP Test 1 and 2 (two-way rmANOVA with *post hoc* Newman Keuls, $p<0.01$). Thus, the conditioning protocol induced place preference that persisted for at least 16 days and was not diminished by repeated testing or post-

conditioning injections of vehicle or the mGluR1 NAM. However, rats treated on days 18 and 19 with the mGluR5 NAMs, MTEP (Fig. 7D, n=15) or MPEP (Fig. 7E, n=9), failed to maintain a preference for the Meth-paired chamber on CPP Test 2 (two-way rmANOVA with *post hoc* Newman Keuls, $p>0.05$). There were no between group differences on CPP Test 2 in horizontal beam breaks (vehicle 3814±27, JNJ16259685 3250±22, MTEP 3640±24, and MPEP 3086±29; $F_{(3)}=1.601$, $p=0.203$) or vertical beam breaks (vehicle 693±82, JNJ16259685 607±61, MTEP 547±38, MPEP 523±74; $F_{(3)}=1.394$, $p=0.257$). These data indicate that mGluR1/5 NAM treatment history did not affect spontaneous motor activity on the subsequent CPP test day.

Discussion

Previous studies have implicated the mGluR1 and 5 in drug-mediated behaviors, but none to-date have determined the role of these receptors in the maintenance of stimulant-associated contextual memories. Acquisition of spatial learning is impaired by administration of JNJ16259685 prior to training on the Morris water maze (Steckler et al. 2005). Yet, mGluR1 NAM treatment after training on the Morris water maze only partially impairs performance on a subsequent test of spatial memory retention (Steckler et al. 2005). Retention of passive avoidance learning is also spared following mGluR1 NAM treatment (Gravius et al. 2005). Though mGluR1 is important for drug reinforcement behaviors, it may not be as critical for the maintenance of spatial memories which play a role in CPP behavior. The current study provided the first evaluation of

mGluR1 NAMs on previously acquired drug-context associations induced by repeated Meth administration. In this paradigm, the mGluR1 NAM JNJ16259685 did not alter subsequent expression of place preference. This outcome may reflect the delayed post-conditioning time of the NAM administration, and it is possible that its administration sooner after place conditioning might result in a disruption of the drug-context association. Supporting this possibility, mGluR1 NAM administration 3 days after repeated cocaine blunted the expression of cocaine-induced motor sensitization (Dravolina et al. 2006). Future studies with chronic or early post-conditioning treatment of JNJ16259685 would further elucidate the involvement of mGluR1 system in maintenance of Meth-associated memories.

Our results suggest that activation of mGluR5 at 13-14 days post-conditioning is necessary for the maintenance of Meth-induced CPP, since rats that were treated with the mGluR5 NAMs MTEP or MPEP failed to demonstrate preference for the Meth-paired context. The use of two mGluR5 NAMs, MTEP and MPEP, (as well as the comparison to the mGluR1 NAM, JNJ16259685) strongly imply that this is an mGluR5-specific phenomenon. The post-conditioning time of mGluR5 modulation may also be important, since pilot data from our lab suggest that an early post-conditioning administration (2 and 3 days after Meth injection) of MPEP does not alter subsequent expression of Meth-induced CPP (i.e., CPP remained statistically significant ($t_{(11)}$=3.292, p=0.007) when tested one day after two daily home cage injections of MPEP (30mg/kg, i.p.)). In Meth self-administering rats that have undergone extinction training,

MTEP pre-treatment reduces cue-induced reinstatement (Gass et al. 2009). Thus, mGluR5 appear to be important for several aspects of Meth reward-mediated behaviors (CPP and self administration) as well as multiple phases of Meth-induced associative learning (expression and maintenance).

Since NAMs act on agonist-bound receptors, results from the current study indicate the importance of glutamate acting on mGluR5 receptors two weeks after acquisition of CPP to sustain the conditioned Meth memory. Therefore, brain regions that highly express mGluR5, such as the NAc, may be key mediators in this behavioral phenomenon (Testa et al. 1994a). Though the literature on glutamate transmitter effects subsequent to Meth administration is limited, increases in NAc glutamate have been shown to occur 3 weeks after repeated Meth (Lominac and Szumlinski 2008). Another possibility is that areas of modest mGluR5 expression, such as the VP, may be characterized by excessive glutamate release following Meth conditioning, resulting in a proportionally large number of agonist-occupied mGluR5. For example, following 14 days after repeated amphetamine, increases in glutamate occur to an acute challenge of the drug in the VP (Chen et al. 2001). Our laboratory has shown that withdrawal from repeated treatment of cocaine results in enhanced response of VP neurons to glutamate (McDaid et al. 2005) and an up-regulation of mGluR5 in the VP following 14 days of withdrawal from Meth (unpublished results). The current study suggests that adaptations in the mGluR5 system occurred approximately two weeks after the acquisition of Meth-induced associative learning.

Cue-elicited relapse is a significant obstacle to abstinence for Meth addicts (Hartz et al. 2001); thus, a pharmacotherapy that can inhibit the maintenance of associative processes should aide in reducing cue evoked drug-seeking. This study has identified a critical time-frame at which negative modulation of mGluR5 receptors is sufficient to disrupt the maintenance of Meth-induced context associations. These novel findings add to current understanding of the neurobiological underpinnings of Meth associated memories and indicate that mGluR5 NAMs deserve further exploration as potential pharmacotherapy for the Meth-withdrawn human addict.

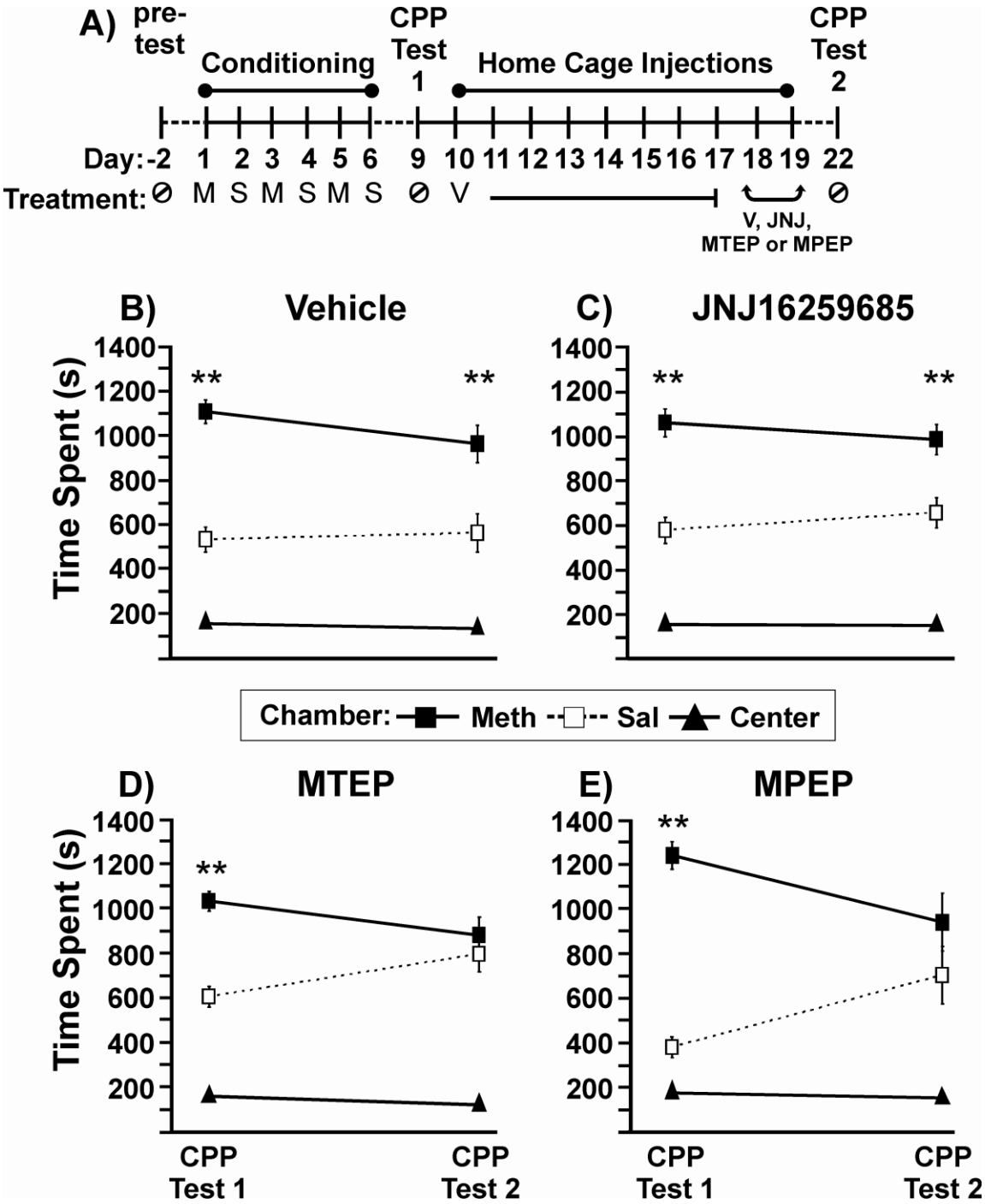

A)
pre-test
Conditioning
CPP Test 1
Home Cage Injections
CPP Test 2
Day: -2 1 2 3 4 5 6 9 10 11 12 13 14 15 16 17 18 19 22
Treatment: ⊘ M S M S M S ⊘ V
V, JNJ, MTEP or MPEP
B) Vehicle
C) JNJ16259685
D) MTEP
E) MPEP
Time Spent (s)
1400
1200
1000
800
600
400
200
**
Chamber: Meth Sal Center
CPP Test 1
CPP Test 2

Figure 7. The mGluR5 NAMs MTEP and MPEP, but not the mGluR1 NAM JNJ1625985, inhibit the expression of Meth-CPP. A) Timeline of treatment and testing. Rats were pre-tested for initial bias on day -2 and subsequently paired with Meth (1mg/kg, i.p.) on days 1, 3, and 5 and saline (1ml/kg, i.p.) on days 2, 4, and 6 for 45min. Chamber preference was assessed on day 9, (CPP Test 1), in a drug-free state in order to verify that the preference developed. On days 18 and 19, rats were given either vehicle (1ml/kg), JNJ1625985 (0.3mg/kg), MTEP (3mg/kg), or MPEP (30mg/kg). Rats were tested for preference three days later (protocol day 22, CPP Test 2) in a drug-free state. **B)** Rats treated with vehicle (n=11) demonstrated a significant difference in amount of time spent in the Meth- *vs.* saline-paired chamber during CPP Test 1 and 2 (significant main effect of chamber $F_{(1,20)}$=23.075, p=0.0001, a non-significant main effect of test $F_{(1,20)}$=0.054, p=0.818, and a significant chamber-test interaction $F_{(1,20)}$=9.099, p=0.007). **C)** Likewise, rats treated with the mGluR1 NAM JNJ1625985 (n=12) spent more time in the Meth- compared to the saline-paired chamber on CPP Tests 1 and 2 (a significant main effect of chamber $F_{(1,22)}$=95.531, p<0.0001, a non-significant main effect of test $F_{(1,22)}$=0.0002, p=0.987, and a non-significant chamber-test interaction $F_{(1,22)}$=0.911, p=0.350). In contrast to these effects, administration of mGluR5 NAMs disrupted the maintenance of Meth-induced CPP. **D)** Rats treated with the mGluR5 NAM MTEP (n=15) demonstrated a significant preference for the Meth-paired chamber during CPP Test 1 but the preference was not observed during CPP Test 2 (a significant main effect of chamber $F_{(1,28)}$=14.686, p=0.001, a non-significant

main effect of test $F_{(1,28)}$=0.088, p=0.769, and a significant chamber-test interaction $F_{(1,28)}$=7.048, p=0.013). **E)** Likewise, rats treated with MPEP (n=9) did not maintain the significant preference for the Meth-paired chamber observed during CPP Test 1 after the day 17 & 18 intervening NAM treatments (a significant main effect of chamber $F_{(1,16)}$=22.785, p=0.0002, a non-significant main effect of test $F_{(1,16)}$=0.024, p=0.879, and a significant chamber-test interaction $F_{(1,16)}$=14.930, p=0.001). CPP data are represented as time spent in the Meth-paired chamber (black solid square with solid black line), saline-paired chamber (white square with dotted black line), or center chamber (black solid triangle with solid black line, not included in statistics but illustrated for qualitative purposes) on CPP Test 1 (left set of data points / column labeled under graph) and CPP Test 2 (right set of data points / column labeled under graph). Data points represent the mean $\pm$ SEM. Repeated measure ANOVA with *post-hoc* Newman Keuls test for multiple comparisons, **p<0.01

CHAPTER V

NOVEL SURFACE EXPRESSION TECHNIQUES FOR *ex vivo* ASSESSMENT OF METABOTROPIC RECEPTORS IN THE MAMMALIAN BRAIN: UTILITY IN MEASURING mGlu5 RECEPTORS AFTER METHAMPHETAMINE-INDUCED ASSOCIATIVE LEARNING

Abstract

Altered expression of receptors and their distribution between the cell membrane surface and the cytoplasm is a means by which psychomotor stimulants alter neuronal transmission. Indeed, ionotropic glutamate receptor distribution is altered in cocaine-sensitized rats, and such changes may contribute to the neuropathology of addictive behaviors. However, stimulant-induced effects on intracellular distribution of metabotropic receptors are not well known. The current study fills this gap by adapting the bis(sulfosuccinimidyl)suberate (BS^3) cross-linking technique, which has been successfully employed to measure changes in ionotropic receptor distribution, to assess surface and intracellular components of metabotropic glutamate for the first time. Metabotropic glutamate receptor subtype 5 (mGluR5) is involved in the behavioral effects of stimulants; including conditioned place preference and motor sensitization.

The current report evaluated if mGluR5 sub-cellular distribution is regulated in the limbic brain regions of rats conditioned with methamphetamine (Meth). Repeated Meth conditioning (1mg/kg) resulted in a significant preference for the Meth-paired context as well as a sensitized motor response. The medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and ventral pallidum (VP) were subsequently assayed for changes in receptor distribution. Meth-conditioned rats demonstrated a significant decrease in the surface to intracellular ratio of mGluR5 in the mPFC, but not the NAc or the VP. This study demonstrates the utility of the BS^3 cross-linking technique to generate an *ex vivo* snapshot of metabotropic glutamate receptor systems after repeated stimulant administration.

Introduction

Receptors located at the neuronal surface serve as targets for extracellularly released neurotransmitters. Internalization renders the receptors unavailable to these transmitters, and this is an important means to regulate neurotransmission. Understanding stimulant-induced changes in receptor distribution may provide insight into neuropathology of stimulant abuse. Ionotropic glutamate receptor subunit surface and intracellular expression can be detected *ex vivo* using the membrane impermeable cross-linking reagent bis(sulfosuccinimidyl)suberate (BS^3). This technique, pioneered by Boudreau and Wolf (Boudreau and Wolf 2005), was utilized to define the role of AMPA receptor subunit surface expression in the synaptic plasticity that occurs

subsequent to repeated cocaine (Boudreau et al. 2007;Boudreau et al. 2009;Ferrario et al. 2010) or amphetamine (Nelson et al, 2009) administration. Metabotropic receptors provide an important means to fine tune excitatory ionotropic transmission and thus likely regulate the maladaptations that occur after repeated stimulant administration.

The behavioral role of these metabotropic receptors has been a topic of great interest, and selective ligands have been used in various rodent models, including conditioned place preference (CPP) and motor sensitization. CPP is a well-established method of measuring the rewarding attributes of abused drugs by assessing the preference for environmental contextual cues previously associated with drug administration (Childs and deWit H. 2009;Tzschentke 1998;Tzschentke 2007). Motor sensitization is the augmentation in motor activity that occurs with repeated administration of a drug, and the brain adaptations associated with this simple behavioral readout may model some of the brain adaptations that occur in the human stimulant abuser (Robinson and Berridge 1993;Stewart and Badiani 1993). Metabotropic glutamate receptors are critical for stimulant-mediated reward (Carroll 2008;Kenny and Markou 2004;Olive 2009;Xi and Gardner 2008) as well as learning and memory processes (Anwyl 1999;Conn and Pin 1997). Specifically, group I subtype 5 metabotropic glutamate receptors (mGluR5), are necessary for the development of Meth- (Miyatake et al. 2005) and expression of amphetamine-induced associative learning (Herzig et al. 2005). These findings underscore that mGluR5 represent a receptor system engaged during stimulant-induced behaviors, and

thus it was selected for our assessments of surface expression following Meth-induced CPP and/or motor sensitization.

Trafficking of mGlu5 receptors is a dynamic process, and a variety of *in vitro* methods have been used to detect surface expression of mGluR5 (Ango et al. 2002;Kumpost et al. 2008;Lee et al. 2008). However, none of these methods allow for the resolution of both surface and intracellular components of metabotropic receptors within the same sample nor had the methods been adapted for *ex vivo* tissue samples. These are important assay attributes for understanding changes imposed by behaviorally relevant treatments of Meth, as well as for future evaluations of the potential of these receptor systems as targets for anti-addiction pharmacotherapies. To fill this gap, we modified the Boudreau and Wolf assay developed for AMPA receptors (Boudreau and Wolf 2005) to provide assessments of metabotropic glutamate receptors. With this assay, the membrane impermeable cross-linking agent, BS^3, binds to extracellular basic residues (*via* an amide bond) of proteins inserted into the membrane, resulting in a high molecular weight aggregate that is easily differentiated from the intracellular protein by using an antibody for protein detection targeted to the carboxy-terminal region of the receptor (Mattson et al. 1993). This high molecular weight aggregate makes up the surface component, and the intracellular component is resolved at the normal molecular weight for the protein of interest within the same lane of the SDS-PAGE gel. The mGluR5 are well suited for this assay since they are part of the group C G-protein coupled receptor family characterized by a large extracellular, amino-terminal domain

where there are many basic amino acids to which the BS^3 agent can bind (Hermans and Challiss 2001;Pin et al. 2003). This assay is also efficient, since ample signal detection is attained without pooling brain tissue samples from multiple animals. These features make this assay ideal for a high through-put, rapid determination of metabotropic receptor distribution that may occur with stimulant reward-mediated behaviors.

The goals of this study were two fold: First, was to adapt the BS^3 cross-linking technique to resolve surface *versus* intracellular pools of mGluR5 within the same *ex vivo* tissue sample. Second, was to apply this assay to test the hypothesis that changes in these receptors would occur in brain regions important for reward and memory taken from rats that exhibit Meth-induced CPP and/or motor sensitization.

Materials and Methods

Animals

A total of 32 male Sprague-Dawley rats weighing between 250-275g at the start of experimentation were used. Rats were acclimated to handling procedures and to the housing *vivarium* (Rush University Medical Center, accredited through the Association for Assessment and Accreditation of Laboratory Animal Care) for at least one week prior to the start of behavioral testing. The *vivarium* was maintained at 23-25°C temperature on a 12hour light cycle (7AM lights on: 7PM lights off) and rats had *ad libitum* access to food and water. Experimentation took place during the light cycle of the rats. During this time,

rats have been shown to produce the most robust CPP performance to amphetamine (Webb et al. 2009). All procedures were approved by the Rush University Institutional Animal Care and Use Committee and were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

Drugs

(+)Methamphetamine hydrochloride (Meth; Sigma-Aldrich, St. Louis, MO) was administered at a dose of 1mg/kg as the base in a 0.9% sterile saline solution. Meth or its vehicle was administered intraperitoneally (i.p.) in a volume of 1ml/kg.

Test Apparatus

Conditioned place preference (CPP) and motor behaviors were quantified in activity boxes that consisted of two large conditioning chambers (25cm x 30cm x 30cm) and a small center chamber (13cm x 30cm x 30cm) (Accuscan Instruments, Inc., Columbus, OH). Behavior in each chamber was measured *via* two banks of photo-beams (24 horizontal and 12 vertical) so as to measure the rats' motor movements in three dimensional space. Beam counts were analyzed with Versamax analyzer and software (Accuscan Instruments, Inc., Columbus, OH). The two large conditioning chambers each had distinct visual (horizontal *vs.* vertical stripes) and tactile (textured floor) cues. One chamber had vertical stripes on the walls and a randomly patterned floor with an overturned paint dish glued in the center (termed Chamber A); the other chamber (i.e., Chamber B) had horizontal stripes on the walls with a square patterned floor and a flat,

rectangular piece of Plexiglas glued in the center; center chamber had solid color walls with a flat, smooth floor. The chambers were separated from each other by removable Plexiglas doors.

Conditioned Place Preference Procedure

Rats were transported from the *vivarium* to the behavioral testing room for habituation at least 30min prior to experimentation. The testing room was dimly lit with white noise continuously present (White noise generator, San Diego Instruments, San Diego, CA). The behavioral paradigm consisted of three phases: pre-test, conditioning, and CPP test (Fig. 8A). For the pre-test, rats were placed in the center chamber; and doors were immediately removed to allow access to the entire CPP box for 15min (900s). Time spent in Chamber A was 348±26s and time spent in Chamber B equaled 459±24s. As time spent in the two conditioning chambers differed ($t_{(31)}$=2.215, p=0.0343), treatment assignments were counterbalanced as follows: half the rats were assigned to receive Meth or saline (day 1) in the chamber in which they spent the greatest amount of time during the pre-test and the other half were assigned to received Meth or saline (day 1) in the chamber in which they spent the least amount of time during the pre-test. Rats were assigned to one of two treatment groups: Meth-conditioned (n=16; Meth administered on days 1, 3, & 5 and saline administered on days 2, 4, & 6) or saline-conditioned (n=16; saline administered on days 1-6). During conditioning (protocol days 1-5, Fig. 8A), rats received the appropriate injection (Meth 1mg/kg or saline 1ml/kg) and were immediately

placed into the appropriate chamber of the CPP box for 45min. Motor activity was assessed throughout the session. To test for the expression of the preference (i.e., CPP test on day 9), drug-free rats were placed in the center chamber; and doors were immediately removed to allow access to the entire CPP box for 15min. Time spent as well as motor activity in each chamber was determined.

Tissue Preparation and Immunoblotting

Rats were decapitated without anesthesia, and mPFC, VP, and NAc tissues (Fig. 10) were harvested within approximately 4min of decapitation one day after the CPP test. The tissue preparation and surface receptor cross-linking methodology was adapted from that of Boudreau and Wolf (Boudreau and Wolf 2005). After being dissected, each brain region was chopped into 400μm slices with a McIlwain tissue chopper (Mickle Laboratory Engineering Co. LTD, Goose Green, UK) and immediately placed into artificial cerebrospinal fluid (aCSF) with 2mM Bis(sulfosuccinimidyl)suberate (BS^3; Thermo Scientific, Rockford, IL). The cross-linking reaction took place for 30min with gentle agitation at 4°C; administration of 100mM glycine terminated the cross-linking reaction (10min at 4°C). The supernatant was separated from the tissue pellet by centrifugation at 14000rpm for 2min (4°C), and the supernatant was removed and the tissue pellet was re-suspended in lysis buffer (25mM HEPES, 500mM NaCl, 2mM EDTA, 20mM NaF, 0.1% Nonidet P-40 (v/v), 1mM DTT, 1mM PMSF, 1 x protease inhibitor cocktail (Calbiochem, La Jolla, CA), phosphatase inhibitor cocktail I (Sigma-Aldrich) and phosphatase inhibitor cocktail II (Sigma-Aldrich). To disrupt the cellular membrane, tissues were sonicated and subsequently

aliquoted and frozen at -80°C for future use. Tissue homogenate samples were prepared with in 1:1 dilution of Laemmli sample buffer (Bio-Rad, Hercules, CA) with β-mercaptoethanol.

For control experiments (Fig. 11), two separate saline treated rats of the same weight from a separate pilot study were prepared without cross-linking (Non-Xlink). Brain regions (Fig. 10) were hand dissected and immediately fast-frozen on dry ice then kept at -80°C until tissue preparation. For tissue preparation, brain regions were Dounce homogenized and sonicated in lysis buffer (25mM HEPES, 1mM EGTA, 1mM EDTA, 100nM okadaic acid, 1mM sodium orthovanadate, 100uM PMSF, 10μg/ml of pepstatin, leupeptin & aprotinin in cocktail form). Protein concentrations were determined via the Bradford method (Bradford 1976) and samples were prepared with sample buffer (NuPAGE LDS Sample Buffer, Invitrogen, Carlsbad, CA) and reducing agent (NuPAGE Sample Reducing Agent, Invitrogen), aliquoted and frozen until use at -80°C.

To allow for immunoblotting, protein samples (20μg) were loaded into 4-15% Tris-HCl gradient gels (Bio-Rad) and electrophoresed at 200V for 45min. The gels were then transferred to a Hy-Bond PVDF membrane (GE Healthcare Limited, Buckinghamshire, UK) for 1.5hr at a current of 1.25A. Nonspecific binding was blocked by incubation of the membrane with TBST (0.05% Tween 20, Sigma-Aldrich), 1% normal goat serum, and 5% non-fat dry milk for at least 1hr. Membranes were then incubated with mGluR5 (1:15,000 in TBS; Upstate, Temecula, CA), or actin (1:20,000 in TBST and 5% non-fat dry milk, Santa Cruz Biotechnology, Inc.) overnight at 4°C. Membranes were washed repeatedly with

TBST and then incubated with the appropriate HRP-conjugated secondary antibody for mGluR5 (goat anti-rabbit 1:15,000 in TBST; Jackson ImmunoResearch, West Grove, PA) and actin (goat anti-rabbit 1:20,000 in TBST and 5% non-fat dry milk; Santa Cruz Biotechnology, Inc.). Membranes were subsequently washed with TBST, TBS, and distilled water.

Chemiluminescent substrate (SuperSignal West Pico, Thermo Scientific, Rockford, IL) was applied to the membrane to visualize the protein of interest on autoradiography film (HyBlot CL, Denville Scientific, Inc., Metuchen, JN). Optical density was determined *via* densitometric analysis with Un-Scan-It Software (Silk Software,Inc., Orem, Utah).

To detect protein levels of Homer1b/c and actin (loading control), the Snap I.D. system (Millipore, Billerica, MA) was used for antibody incubation and washing. After samples were electrophoresed on 10% acrylamide Tris gels and transferred onto PVDF membranes as stated above, membranes were blocked for nonspecific binding with filtered 0.5% non-fat dry milk in TBS-T (0.1% Tween-20) then vacuumed through the Snap I.D. Primary antibody for mGluR5 (0.5µl/ml; Upstate), Homer 1b/c (2.67µl/ml; Millipore) or actin (0.33 µl/ml, Santa Cruz Biotechnology) was then applied and incubated on the membranes for 10min and subsequently vacuumed through the Snap I.D. system. Membranes were incubated with the appropriate HRP-conjugated IgG secondary antibody: goat anti-rabbit (actin 0.33µl/ml; Jackson) and rabbit anti-rat (Homer1b/c 1.33µl/ml, Millipore). After vacuuming through the secondary antibody,

membranes were washed and exposed to chemilumenescent substrate as described above.

For pre-absorption assays, immunoblotting was conducted as stated above. The PVDF membrane was cut in half and antibody plus the blocking peptide (pre-incubated for 1hr at room temperature) was applied to half the other half was processed as described previously; membranes were incubated overnight at 4°C. The mGluR5 synthetic blocking peptide (1:500 in 1xTBS; Neuromics Antibodies, Edina, MN) used was specific to the epitope of the anti-mGluR5 rabbit immunoaffinity purified IgG primary antibody (1:15,000 in 1xTBS; Upstate). Immunoblots were subsequently washed, incubated with the appropriate secondary antibody, and developed as described previously.

Statistics

CPP: Preference was considered to have been achieved when a significantly greater amount of time was spent in the Meth-paired compared to the saline-paired chamber during the CPP test; paired *t*-test, (α=0.025). Development of motor sensitization was determined by a significant increase in activity between the first (conditioning day 1) and last (conditioning day 5) injection of drug; paired *t*-test (α=0.05). Immunoblotting: Optical density of the entire smear of surface signal component (as pictured in Fig. 12, denoted S) and intracellular component (I) were determined and a ratio was calculated (S/I ratio) by dividing the S by I. Total protein was calculated by adding the S and I components and dividing this value by the actin loading control. The individual S and I components were calculated by dividing the optical density of S and I by the

loading control, actin. The S/I ratio, total protein, S and I values were normalized to the average saline treatment groups within each gel and multiplied by 100 to determine Optical Density (% Average Saline) as shown in Fig. 11. Student's *t*-tests (α=0.05) were used to determine differences between Meth and saline treatment groups in surface/intracellular (S/I) ratio, total protein, surface (S) component and intracellular (I) component for mGluR5. All data are represented as mean ± standard error of the mean (SEM). Statistical outliers were determined as more than two standard deviations above or below the mean for any data set. For the immunoblotting data, 95 out of 720 data points were removed. Four out of 128 data points were removed in the motor sensitization data, and no outliers were removed from the CPP data.

Results

The conditioning protocol (Fig. 8A) successfully induced a preference for the Meth-paired chamber in rats that were Meth-conditioned ($t_{(15)}$=4.489, p=0.0004; Fig. 8C) that was not evident in rats that were treated with saline ($t_{(15)}$=1.063, p=0.3044, Fig. 8B). The repeated administration of Meth (1mg/kg) lead to a significant increase in horizontal activity ($t_{(15)}$=2.611, p=0.0197), and vertical time ($t_{(15)}$=2.250, p=0.0399) between the first (conditioning day 1) and last (conditioning day 5) administration (Fig. 9A,B). Rats treated repeatedly with saline (1ml/kg), however, demonstrated a significant decrease in horizontal activity ($t_{(13)}$=3.027, p=0.0097) and vertical time ($t_{(13)}$=5.160, p=0.0002) to the fifth compared to the first injection (Fig. 9C,D). These data indicate that rats

given repeated, intermittent Meth developed motor sensitization, while rats given saline habituated to the environment in which it was administered.

A pre-absorption assay validated antibody specificity for the mGluR5 primary antibody used for the immunoblotting experiments (Fig. 11A-C). In this experiment, pre-absorption with the respective blocking peptide completely inhibited binding of the antibody in tissue with (Xlink) and without (Non-Xlink) the BS^3 cross-linking reagent (Fig. 11B). Shown in Fig. 11 is NAc for mGluR5. Comparison of Xlink and Non-Xlink tissue demonstrated that the high molecular weight aggregate (which corresponds to the BS^3 bound receptor inserted into the surface of the membrane) was not present in the Non-Xlink tissue and that the molecular weights of the intracellular components for the mGluR5 (Fig. 11A) were found at the expected molecular weight in both Xlink and Non-Xlink tissue (mGluR5 molecular weight approximately 130kD). Thus, the cross-linking procedure allows for the simultaneous detection of the high molecular weight aggregate inserted into the membrane surface *versus* receptors that are located in the intracellular compartment of the cell. To validate that BS^3 did not permeate the cell membrane, we used cross-linked tissue to evaluate Homer 1b/c, a protein that is located exclusively in the intracellular compartment. Immunoblots of Homer 1b/c resulted in a discrete band at approximately 47kD which correctly corresponds with the molecular weight of Homer 1b/c and no high molecular weight aggregate was detected (Fig. 11C).

Evaluation of distribution of mGlu5 receptors revealed excellent detection levels in all brain regions tested (i.e., mPFC, NAc and VP). While no changes

were observed for the mGluR5 receptor in the NAc (S/I ratio, $t_{(28)}$=0.346, p= 0.731; total protein, $t_{(29)}$=1.96, p=0.059; surface, $t_{(29)}$=1.342, p=0.190; intracellular, $t_{(29)}$=0.2235, p=0.8247, Fig. 12B) or the VP (S/I ratio, $t_{(29)}$=1.33, p=0.195; total protein, $t_{(29)}$=0.265, p=0.793; surface, $t_{(29)}$=0.7878, p=0.4372, intracellular, $t_{(28)}$=1.635, p=0.1133, Fig. 12C), Meth-conditioning significantly reduced the mPFC mGluR5 S/I ratio ($t_{(28)}$=3.14, p= 0.004) and increased the intracellular component ($t_{(30)}$=2.98, p=0.005) without altering total protein ($t_{(29)}$=0.11, p= 0.909) or the surface component ($t_{(29)}$=0.3836, p=0.7041; Fig. 12A). Thus, brain and receptor specific changes were observed in the S/I ratio of the mGluR5 in the mPFC of Meth-conditioned rats.

4. Discussion

We demonstrated for the first time that *ex vivo* BS^3 cross-linking methodology can be applied to metabotropic glutamate receptors. We also revealed that this methodology can detect brain region-selective differences in responding to Meth in behaviorally relevant doses. That is, in the mPFC, the S/I ratio and intracellular levels of mGluR5 was significantly reduced after repeated Meth administration that induced CPP and motor sensitization.

Distribution of mGluR5 can be determined using a variety of techniques. Biotinylation has been successfully employed *in vitro* (Lee et al. 2008); however, this technique may produce highly variable results because intracellular and surface components are quantified in separate gels. Synaptosomal fractionation has also been used to determine the distribution of mGluR5 receptors *ex vivo*

(Ghasemzadeh et al. 2009a;Ghasemzadeh et al. 2009b;Shaffer et al. 2010); however, this technique does not specifically determine levels of receptor protein inserted into the surface of the cellular membrane. The technique used in the current study, pioneered by Boudreau and Wolf (Boudreau and Wolf 2005) to evaluate the surface expression of AMPA receptor subunits, provides several advantages. The membrane impermeable cross-linking agent BS^3 binds to extracellular basic residues of proteins inserted into the membrane resulting in a high molecular weight aggregate that is easily differentiated from the intracellular protein by using an antibody for protein detection targeted to the carboxy-terminal region of the receptor. We validated the lack of intracellular BS^3 binding using Homer 1b/c as an intracellular control since this is a scaffolding protein is important for trafficking of the mGluR5 receptor (Ango et al. 2002;Roche et al. 1999). This finding is in keeping with the work of Boudreau and Wolf who demonstrated discrete, monomeric banding of tyrosine hydroxylase in BS^3 cross-linked tissue (Boudreau and Wolf 2005). It is important to note that although this assay has been used to monitor changes in the expression and distribution of mGluR5, AMPA receptors (Boudreau et al. 2007;Boudreau and Wolf 2005;Conrad et al. 2008;Mickiewicz et al. 2006;Nelson et al. 2009), and now dopamine receptors (Conrad et al. 2010), it does not work for all proteins inserted into the surface of the membrane. BS^3 binds to basic amino acid residues in the N-terminal region, and there must be an adequate amount of these residues to allow for differentiation of the surface and intracellular components. The mGluR5 are part of the class C family of

metabotropic receptors that have very large extracellular domains, which each contain many basic amino acid residues (Hermans and Challiss 2001;Pin et al. 2003). Another critical technical consideration relates to the fact that BS^3 binds to the amino (N)-terminal of the receptor. Consequently, it can mask antibody binding sites located in the N-terminus; and so an antibody that binds to the intracellular carboxy-terminal region of the receptor must be used.

In the current study, we observed that in the mPFC of rats showing Meth-induced CPP, the mGluR5 S/I ratio was decreased compared to saline control tissue, but the total mGluR5 protein between Meth and saline groups was not different. This outcome indicated a redistribution of mGluR5 to the intracellular pool of the cell, which is in accord with the significant increase in the intracellular pool of mGluR5 receptors that we observed for the mPFC. Thus, it appears that cells within the mPFC have adapted to the Meth conditioning treatment such that there are fewer mGluR5 on the surface and more in the intracellular pool.

The decreased S/I ratio and increase in intracellular levels of the mGluR5 receptor in the mPFC may be a compensatory mechanism to counteract the hyperexcitable state after repeated Meth administration. Meth administration increases extracellular glutamate levels in the mPFC (Shoblock et al. 2003) and this effect is enhanced by repeated pairing of Meth with environmental cues (Qi et al. 2009). Also, mGluR5 receptors are desensitized with prolonged agonist exposure (Catania et al. 1991). Shaffer and colleagues found that an acute treatment of amphetamine increases synaptosomal mGluR5 in the mPFC (Shaffer et al. 2010) whereas glutamate levels are unchanged in this region

following amphetamine treatment (Shoblock et al. 2003). One day after repeated cocaine, a time frame at which glutamate levels are increased in response to an additional cocaine injection (Williams and Steketee 2004), an increase in mGluR5 in the mPFC is observed in the synaptosomal fraction (Ghasemzadeh et al. 2009b). The divergent outcomes found in the current study may reflect drug-specific effects (Meth *vs*. cocaine), withdrawal time specific effects (four days *vs*. one day) or dissection-dependent effects (ventral *vs*. dorsal mPFC). This change may also reflect the response to re-exposure to conditioning cues 24hr before the tissue was collected. The current study adds to the literature to demonstrate that repeated Meth administration, which was sufficient to induce CPP and MSn, may be important for some of the adaptations that contribute to the addiction phenomenon.

No other significant changes in the expression or distribution of the mGluR5 receptor were identified in the NAc or VP. This was an unexpected result, as there are a few examples of dynamic effects of mGluR5 expression in the NAc in response to other stimulants, cocaine and amphetamine (the VP had not been previously studied). For example, a repeated, sensitizing amphetamine administration results in a decrease in mGluR5 mRNA in the NAc that occurs 3hr after drug administration and persists up to 28 days (Mao and Wang 2001). A potential explanation for this disparate finding from our study is that there is an increase in extracellular glutamate levels in the NAc following amphetamine but not Meth administration (Shoblock et al. 2003). Therefore, mGluR5 receptors may have internalized in response to repeated glutamate exposure after repeated

amphetamine which would not occur with Meth administration. In response to repeated cocaine administration, on the other hand, synaptosomal protein levels of mGluR5 are unchanged at early, and increased following extended (3 week) withdrawal (Ghasemzadeh et al. 2009a). Yet, Swanson and colleagues demonstrate a decrease in total mGluR5 protein and its associated scaffolding protein Homer 1b/c at the same withdrawal time from repeated cocaine (Swanson et al. 2001). Thus, it appears that glutamate receptor adaptations are differentially altered by the various stimulant drugs. There is precedence for this conclusion as Wolf and colleagues found differential adaptations in the surface expression of AMPA receptor subunits to repeated cocaine (increase in GluR1surface expression) and amphetamine (no change in GluR1 levels) administration in the NAc using the BS^3 cross-linking assay (Boudreau et al. 2007;Nelson et al. 2009). As little is known about sub-cellular dynamics of mGluR5, following behaviorally relevant Meth administration, our results will aid in a more thorough characterization of mGluR5 expression in the NAc in response to stimulants. We have also extended the literature by including the VP in our assessments. Though mGluR5 levels in the VP were unaltered in the current study, these findings are an important step in the characterization of a brain region known to be involved in motivation to action and incentive salience.

The current study has demonstrated the application of the BS^3 cross-linking technique to assess surface expression of mGluR5 which provides an *ex vivo* snapshot of the brain of *in vivo* Meth-induced processes. We have identified brain region specific alterations in surface expression of mGlu5 receptor and

exciting potential of this work would be to determine if this receptor redistribution is functionally relevant in electrophysiological assays.

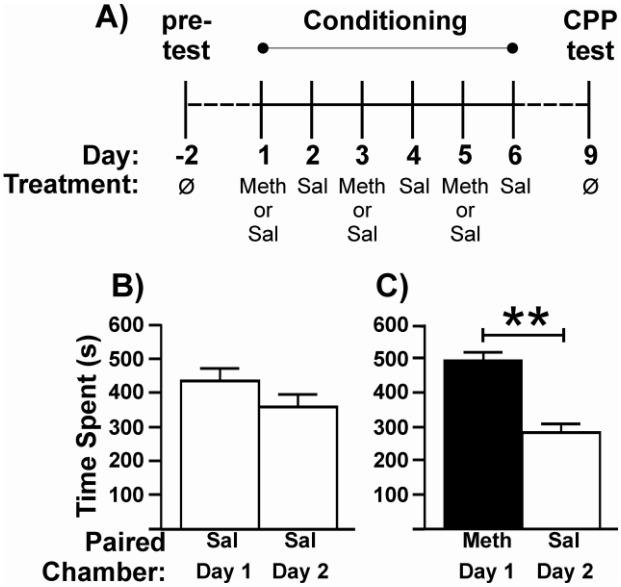


Figure 8. Meth-induced CPP. A) Time-line of behavioral protocol. For the pre-test (day -2), drug-free rats were allowed to explore the entire box for 15min. Conditioning occurred for six days; for Meth conditioned rats, Meth (1mg/ml/kg, i.p.) was paired with one chamber of the box on days 1, 3, & 5, and the saline (1ml/kg, i.p.) was paired with the opposite chamber on days 2, 4, & 6). Saline conditioned rats were treated in the same manner but saline was administered on all days (days 1-6). Conditioning sessions were 45min in duration. Three days later (day 9), rats were tested for the expression of Meth-induced CPP in a drug-free state (15min). Meth, methamphetamine, Sal, saline, Ø, no drug. **B)** Rats conditioned with saline (n=16) did not demonstrate a preference for either chamber on the CPP Test day (Paired *t*-test, $p>0.025$). **C)** Rats conditioned with Meth (n=16) spent significantly more time in the Meth-paired (dark bar) *vs*. the saline-paired (white bar) chamber during the CPP Test (Paired *t*-test, ***$p<0.001$).

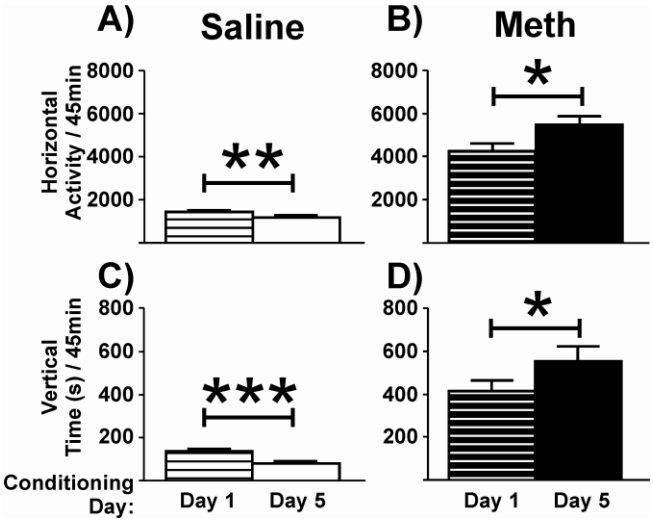


Figure 9. Repeated administration of methamphetamine was sufficient to induce the development of motor sensitization. Horizontal Activity (number of horizontal beam breaks) in **A)** Saline (n=14)- and **B)** Meth (n=16)-conditioned rats. Vertical Time (sec spent breaking beams in the vertical plane) in **C)** Saline-conditioned (n=14) and **D)** Meth-conditioned (n=16) rats. Shown here are mean ± SEM for data obtained on conditioning day 1 and conditioning day 5 of the behavioral paradigm (treatment protocol illustrated in Fig. 1A). Paired *t*-test, *p<0.05, **p<0.01, ***p<0.001.

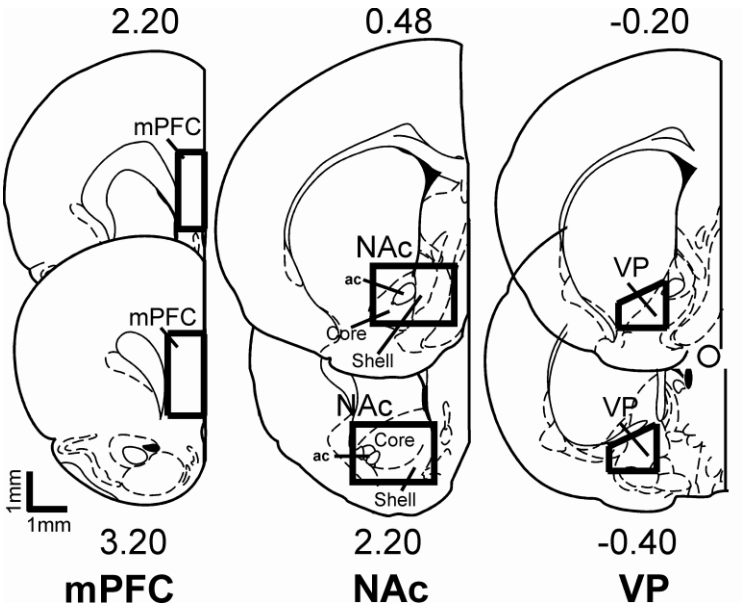


Figure 10. Stereotaxic brain maps indicating regions dissected. This figure is modified from Paxinos and Watson (1998) with numbers indicating the distance in millimeters from Bregma. The boxes indicate dissections from the mPFC, medial prefrontal cortex, NAc, nucleus accumbens, and VP, ventral pallidum.

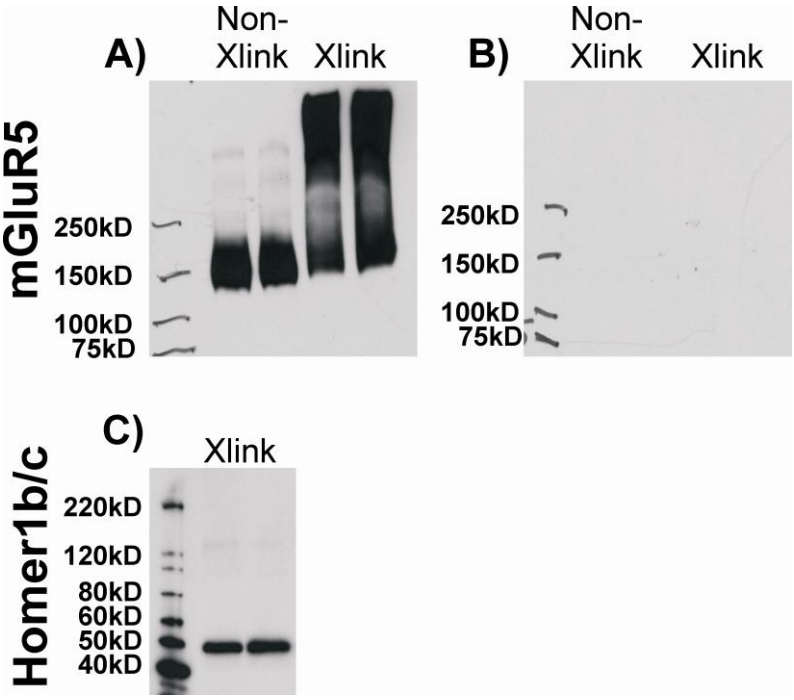


Figure 11. Validation of BS^3 cross-linking technique applied to mGluR5. **A)** Cross-linked (Xlink) and non-cross-linked (Non-Xlink) NAc tissues probed for mGluR5 demonstrated mGluR5 receptor protein at ~130kD, corresponding to intracellular protein, and a high molecular weight aggregate at >400kD, corresponding to surface receptor bound to BS^3 which was present in Xlink tissue only. An overexposed blot is pictured here as to demonstrate both intracellular and surface proteins on the same exposure. Also, the same exposure was demonstrated in **B)** for pre-incubation of the antibody with the blocking peptide that inhibited all antibody binding, indicating antibody specificity demonstrating no signal at longer exposures. **C)** A protein that is exclusively located in the intracellular cell compartment, Homer1b/c (an intracellular scaffolding protein) was detected at 47kD without any high molecular weight

aggregate signal indicating that that BS^3 cross-linker is not penetrating cellular membranes.

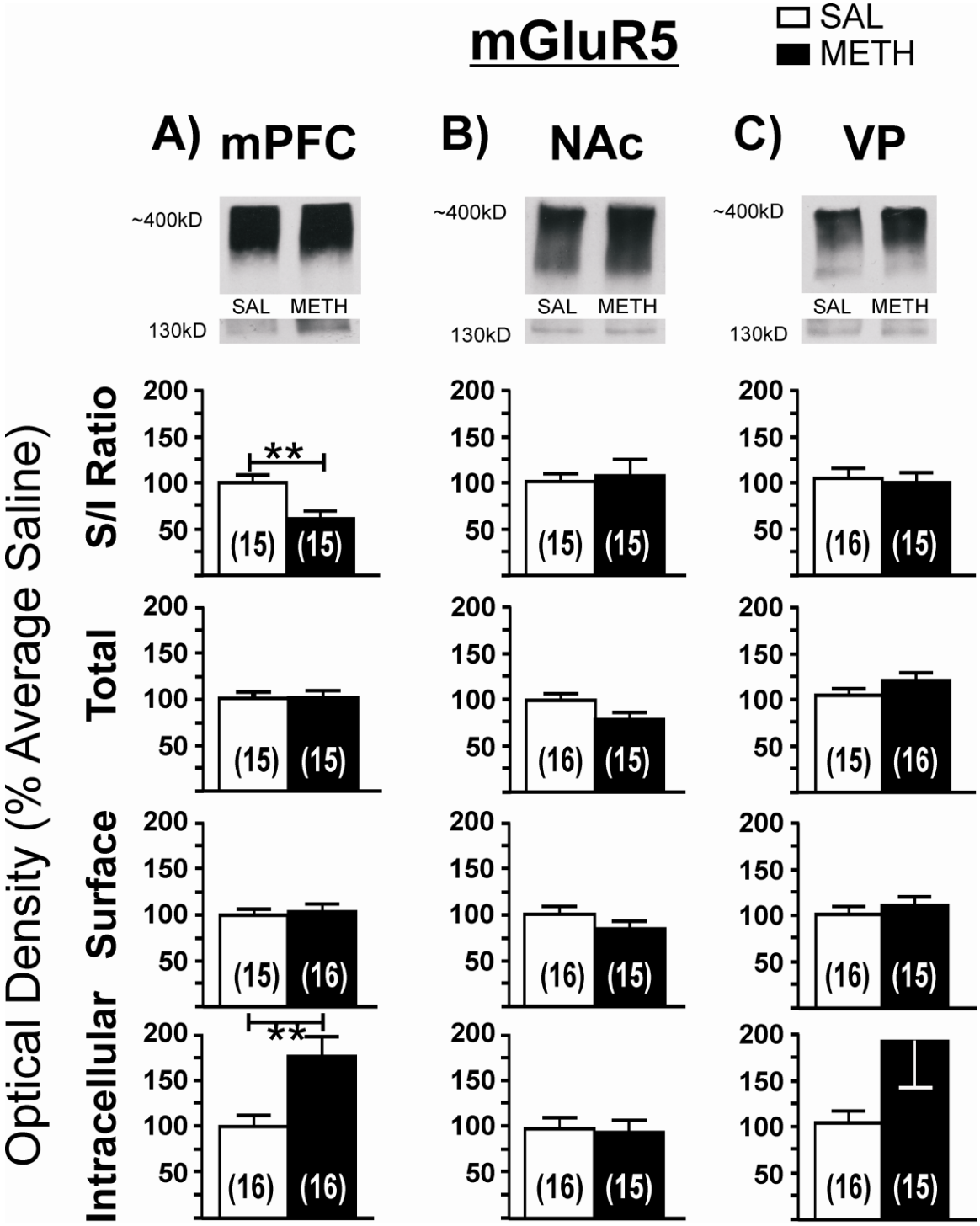

mGluR5
SAL
METH
A) mPFC
B) NAc
C) VP
~400kD
130kD
SAL METH
Optical Density (% Average Saline)
S/I Ratio
Total
Surface
Intracellular
200
150
100
50
**
(15) (15)
(15) (15)
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Figure 12. mGluR5 S/I ratio was decreased in the mPFC of Meth-conditioned rats. Treatment group is indicated by key: Sal=saline conditioned (open bar), Meth=methamphetamine conditioned (filled bar). Representative immunoblots are illustrated above each set of bar graphs. Data are shown for surface to intracellular ratio (S/I Ratio), total protein (Total), surface and intracellular receptor components. **A)** There was a significant reduction in the S/I ratio of Meth-treated rats that expressed CPP in the mPFC. There was no change in total mGluR5 or surface protein in the mPFC. There was also a significant increase in the intracellular component of mGluR5 in the mPFC. There were no changes in mGluR5 S/I ratio, total protein, surface or intracellular protein components in the **B)** NAc or **C)** the VP. Student's *t*-test, **p<0.01.

CHAPTER VI

DIFFERENTIAL DISTRIBUTION OF IONOTROPIC AND METABOTROPIC GLUTAMATE RECEPTORS FOLLOWING ACUTE AND REPEATED ADMINISTRATION OF MORPHINE OR METHAMPHETAMINE: ROLE OF mGluR5 IN EXPRESSION OF GluR2 AND STEP61 PROTEINS

The current chapter is the result of collaboration with Dr. Amanda Mickiewicz, a former student in the Napier laboratory. Dr. Mickiewicz, as a part of her dissertation project, completed the work involving opiates. In alignment with the body of this dissertation work, only results of this chapter dealing with methamphetamine will be addressed in the General Discussion.

Abstract

The metabotropic glutamate group I subtype 5 receptor (mGluR5) is critical for neuronal and behavioral effects of opiates and stimulants including the progressive enhancement (sensitization) of motor activity in laboratory rats. The mGluR5 also regulates surface expression of ionotropic AMPA receptor subunits through the activation of striatal enriched tyrosine phosphatase isoform 61 ($STEP_{61}$).

To best study this phenomenon, we measured AMPA subunit and mGluR5 distribution levels in the same tissue samples harvested from rats after induction of motor effects or after sensitized responding to the opiate morphine and the stimulant methamphetamine (Meth). Application of the bis(sulfosuccinimidyl)suberate (BS^3) cross-linker to medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and ventral pallidum (VP) tissue collected from rats one day after acute, or fourteen days after repeated morphine or Meth, allowed for detection of AMPA receptor subunits (GluR1 and GluR2) and mGluR5 surface expression as well as intracellular $STEP_{61}$ protein via Western blot analysis. Surface expression of GluR1 and GluR2 were decreased in the mPFC following acute morphine. Fourteen days after a sensitizing regimen of repeated morphine or Meth, the surface expression to intracellular protein ratio of GluR2 in the mPFC and mGluR5 in the VP was increased. In repeated, morphine-treated rats, mGluR5 surface expression decreased without a change in $STEP_{61}$ in the mPFC. In Meth treated rats, mGluR5 levels were not altered; however, STEP61 was decreased. We hypothesized that function of mGluR5 was responsible for the alterations that occurred in GluR2 and $STEP_{61}$. After administering an mGluR5-selective negative allosteric modulator, MTEP, prior to Meth in a separate group of animals, levels of GluR2 and $STEP_{61}$ were not altered in the mPFC after fourteen days of withdrawal. These data give an excellent comparison of glutamate receptor adaptations that occur subsequent to opiate and stimulant sensitization. Furthermore we provide evidence that mGluR5 is

functionally responsible for GluR2 and $STEP_{61}$ alterations induced by methamphetamine sensitization.

Introduction

Repeated administration of opiates and stimulants enhances motor activity beyond that obtained with a single injection, a phenomenon often referred to as sensitization. Drug-induced behavioral and neuronal sensitization can persist long after the cessation of drug administration. The neuronal changes associated with sensitized behaviors in rats may model some of the persistent brain adaptations that occur in the abstinent, but drug-addicted human (Paulson et al. 1991;Robinson and Berridge 1993;Sax and Strakowski 2001;Stewart and Badiani 1993). The neuronal circuitry that undergoes sensitization to both opiates and stimulants involves the limbic system, including glutamatergic projections from the medial prefrontal cortex (mPFC), to the nucleus accumbens (NAc) (Christie et al. 1985) and the ventral pallidum (VP) (Carnes et al. 1990;Fuller et al. 1987;Sesack et al. 1989). However, these brain structures are involved in different phases of the sensitization processes. The mPFC is implicated in the development of behavioral sensitization (Bjijou et al. 2002;Cador et al. 1999;Pierce et al. 1998;Wolf et al. 1995;Wolf and Xue 1999), while the NAc mediates the maintenance and expression of the behavior (Pierce and Kalivas 1997;Vanderschuren and Kalivas 2000). Studies from our laboratory demonstrate that the VP is critical for the development, maintenance and expression of sensitization (Dallimore et al. 2006;Johnson and Napier

2000;McDaid et al. 2005;McDaid et al. 2006a;Mickiewicz et al. 2009). Glutamatergic transmission in each of these brain regions is altered following repeated treatment of opiates and stimulants, and most of the evaluations of neuronal sensitization show that alterations in the mPFC occur during development; the NAc is altered during maintenance and expression, whereas glutamatergic enhancement occurs in the VP during all three phases of sensitization (Chen et al. 2001;Hao et al. 2007b;Johnson and Napier 1997;LaLumiere and Kalivas 2008;McDaid et al. 2005;McDaid et al. 2006a;Pierce et al. 1996;Qi et al. 2009). Thus, to best understand the complexities of drug-induced sensitization, an appreciation of the temporal (i.e., sensitization phase) and spatial (i.e., brain region) effects of the glutamatergic processes need to be considered.

Group I subtype 5 metabotropic glutamateric receptors (mGluR5) are located throughout the limbic circuitry (Lu et al. 1999;Romano et al. 1995;Testa et al. 1994b), and these receptors are critical for both opiate- and stimulant-mediated behaviors, including sensitization (Gass et al. 2009;Herzig et al. 2005;Herzig and Schmidt 2004;Kotlinska and Bochenski 2007;Miyatake et al. 2005). mGluR5 regulates release of glutamate and the functional state of the ionotropic receptors. For example, activated mGluR5 induces internalization of AMPA receptors containing the GluR1 and GluR2 subunits (Snyder et al. 2001) *via* de-phosphorylation of AMPA receptor subunits by the striatal-enriched protein tyrosine phosphatase, isoform 61 ($STEP_{61}$) (Zhang et al. 2008). $STEP_{61}$ is expressed in CNS neurons, including those in striatal and cortical regions

(Boulanger et al. 1995;Bult et al. 1996). Blockade of STEP was recently shown to reduce the development of amphetamine-induced behavioral sensitization (Tashev et al. 2009). Therefore, we considered that mGluR5 would be involved in the regulation of AMPA receptor surface expression and $STEP_{61}$ expression in brain regions where these proteins were altered by opiates and/or stimulants.

Regulating the number of receptors available for transmitter activation is an important means for regulating function, and processes involved in this regulation are important underpinnings of drug-induced neuroplastictiy. Increases in AMPA receptor surface expression enhance synaptic strength, which promotes both the development and the maintenance of drug-induced sensitization (Kauer and Malenka 2007;Wolf et al. 2004). Little is known about these processes during opiate-induced sensitization, and the psychostimulant most studied is cocaine. With repeated, once daily treatments of cocaine, one day after the last injection (a time when the animal would be 'expecting' a subsequent cocaine treatment) there is little change in GluR1, GluR2 and mGluR5 levels in the mPFC and NAc(Ghasemzadeh et al. 2009a;Ghasemzadeh et al. 2009b); the VP was not assessed in these studies. Following extended (21 days) withdrawal from repeated once-daily cocaine administration, there is an overall up-regulation of these receptor proteins in synaptosomal membrane fractions in the NAc and mPFC, as well as enhanced surface expression of GluR1 and GluR2/3 subunits (Boudreau and Wolf 2005;Ghasemzadeh et al. 2009a;Ghasemzadeh et al. 2009b). Extended withdrawal from repeated amphetamine, on the other hand, does not result in altered surface expression of GluR1 or GluR2 in the NAc

(Nelson et al. 2009). These results indicate that the effects of one stimulant on glutamate receptor trafficking cannot be generalized to all stimulants.

The current study was designed to determine if AMPA receptor surface expression in limbic brain regions was commonly regulated by the opiates and stimulants, if this co-varied with mGluR5 surface expression and STEP levels, and if the apparent trafficking processes involved in induction of behavioral sensitization were common to those involved in the maintenance. Furthermore, we were able to determine if the mGluR5 receptor mediated these drug-induced receptor adaptations *via* administration of an mGluR5 negative allosteric modulator (NAM). Toward those objectives, we used a new methodology that allows for *ex vivo* assessments of surface expression of receptor proteins in brain samples taken from rats subjected to once-daily treatments of the opiate, morphine and the stimulant, methamphetamine (Meth) (Mickiewicz & Napier, *under review;* Herrold *et al., under review*). To provide a temporal and spatial snapshot of maintenance, we harvested the mPFC, NAc and VP following 14 days of withdrawal, a time frame that we have observed maintenance-related effects in these brain regions in both morphine- and Meth-treated rats (McDaid et al. 2005;McDaid et al. 2006a;McDaid et al. 2006b;Mickiewicz et al. 2009). To ascertain if similar changes in surface expression may be part of the induction process, we also assayed *ex vivo* brain samples taken one day after a single drug treatment (i.e., at a time when rats in the repeated treatment protocol received a second drug treatment).

The experimental outcomes significantly moved the field forward by filling several gaps in the literature, include the following "firsts": i) *Characterization of surface expression of both AMPA receptor subunits, as well as mGluR5, following a sensitizing regimen of both an opiate and a stimulant*. ii) *Inclusion of the VP in each of these assessments*. iii) *Determination of a mechanism for mGluR5 regulation of AMPA receptor subunit surface expression subsequent to drug-induced sensitization*. iv) *The ability of the negative allosteric modulator MTEP to block both the behavioral and neuronal sensitization induced by Meth*. These data are particularly informative since surface expression of mGluR5 has not been assessed following morphine administration, and the effects of Meth administration on AMPA receptor proteins or surface expression of mGluR5 have not been studied in *ex vivo* brain tissue samples.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 200-225g upon arrival (Harlan, Indianapolis, IN) were housed in pairs under environmentally controlled conditions (7:00AM/7:00PM light/dark cycle, temperature maintained at 23-25^{o}C) with *ad libitum* access to rat chow and water. The rats were habituated to vivarium conditions for at least one week prior to experimentation. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington DC) and were

approved by the Institutional Animal Care and Use Committees at Loyola and Rush University Medical Centers.

Drugs

Morphine sulfate, obtained from the National Institute on Drug Abuse (Bethesda, MD), was dissolved in 0.9% saline to yield a dose of 8mg/ml/kg as the salt. Meth hydrochloride (Sigma, St. Louis, MO) was dissolved in saline to yield a dose of 1mg/ml/kg as the salt. Control rats received the saline vehicle (1ml/kg). Injections were given subcutaneously (sc). 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP, Tocris Bioscience, Ellisville, MO) was dissolved in sterile water containing 20% w/v 2-hydroxypropyl-β-cyclodextrin (Sigma) to yield a dose of 5mg/ml/kg as the salt. MTEP and its vehicle were injected intraperitoneally (ip).

Behavioral Assessments and Treatment Protocols

Rats were transported across the hall from the housing room to the test room at least 30min prior to the start of the experiment. The test room was dimly lit (5 -10 foot candles) with white noise continuously present. Two days prior to behavioral assessment, the rats were habituated to the activity boxes for 1hr, given sc saline injections, and motor activity was monitored for 2hr post-injection. The motor score obtained on the second day was used as baseline activity. Drug treatments began one day after baseline collection; rats were randomly assigned to receive saline, morphine or Meth. Motor activity was

collected using Plexiglas activity chambers (25cm x 30cm x 30cm) equipped with two sets of photobeams (AccuScan Instruments, Inc., Columbus, OH) that allowed behavioral quantification in three dimensional space. Rats were allowed to habituate to the motor boxes for 1hr, given a sc injection of the drug (saline, morphine, Meth) and then immediately placed back into the motor box for 3hr of behavioral monitoring. For the acute treatment protocol, On Day 1, 32 rats were administered the drugs (morphine, Meth or the saline vehicle) and motor activity was quantified. Rats were sacrificed the following day (Day 2) *via* rapid decapitation to collect tissue for biochemical analysis (see below). For the repeated treatment protocol, 64 rats were administered saline, morphine or Meth once-daily for three days (Days 1-3), and motor activity was quantified. Following a 14 day withdrawal (W/D) period, a subset of rats (n=32) was tested for expression of sensitization after administration of morphine or Meth. Other rats (n=32) were not given a drug challenge and were sacrificed *via* rapid decapitation on W/D 14 to collect tissue for biochemical analysis.

To assess the effects of the mGluR5 NAM, MTEP on repeated Meth treatments, rats were given vehicle (20% w/v 2-hydroxypropyl-β-cyclodextrin) or the mGluR5 NAM, MTEP 10min before saline (n=12) or Meth (n=10), respectively, once-daily for three days (Days 1-3) and motor activity was quantified. On W/D day 14, rats were sacrificed *via* rapid decapitation to collect tissue for biochemical analysis.

Protein Cross-Linking with BS^3

The brains were removed within 45-60sec after decapitation and immediately chilled in ice-cold artificial cerebrospinal fluid. The mPFC, NAc, and VP were dissected (see Fig. 13) and chopped into 400μm slices using a McIlwain tissue chopper (The Vibratome Company, O'Fallon, MO). Methods for receptor cross-linking were based on Boudreau & Wolf (2005). Briefly, the slices were quickly transferred to centrifuge tubes containing artificial CSF and 2mM BS^3 (Pierce, Rockford, IL) and incubated for 30min on a rocker at 4°C. The cross-linking reaction was terminated by the addition of 100mM glycine for 10min at 4°C. The slices were pelleted by 2min of centrifugation and the supernatant discarded. The pellets were re-suspended in ice-cold lysis buffer [25mM HEPES, pH 7.4, 500mM NaCl, 2mM EDTA, 1mM DTT, 1mM PMSF, 20mM NaF, 1x phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), 1x protease inhibitor cocktail (Calbiochem, La Jolla, CA), and 0.1% Nonidet P-40]. The samples were sonicated for 5sec, centrifuged for 2min, aliquotted, and stored at -80°C until analysis. Total protein concentration of lysates was determined according to the Bradford method (Bradford 1976).

Immunoblotting GluR1, GluR2, mGluR5 and STEP

Samples (20μg) were loaded and electrophoresed on 4-15% gradient Tris-HCl gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes for immunoblotting. Non-specific binding sites were blocked using 1% normal goat serum and 5% nonfat dry milk in TBS-Tween 20 (TBS-T, Sigma), pH 7.4 for 1hr

at room temperature. Membranes were incubated in primary antibody (GluR1, 1:1000, Millipore, Billerica, MA; mGluR5, 1:15,000, Millipore; GluR2, 1:4000, Millipore; STEP, 1:4000, Millipore; actin, 1:20,000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4^{o}C with gentle shaking. Following six washes for 5min each in TBS-T, membranes were incubated in HRP-conjugated secondary antibody (goat anti-rabbit; either 1:10,000 (Millipore, GluR1 and GluR2), 1:15,000 (Jackson ImmunoResearch, West Grove, PA, mGluR5), or 1:20,000 (Jackson, actin); rabbit anti-mouse, 1:4000 (Jackson, STEP)) for 1hr at room temperature. Membranes were again washed in TBS-T (six x 5min), immersed in enhanced chemiluminescent substrate (Pico, Pierce, Rockford, IL) for 5min, and exposed to HyBlot CL film (Denville Scientific, Metuchen, NJ). Two bands were visualized on film; a dense, high molecular weight band of ~400-600 kDa (cross-linked GluR1 or mGluR5 corresponding to the surface pool (S)) and a band at ~106 kDa (GluR1 and GluR2), ~135kDa (mGluR5), (i.e., unmodified GluR1, GluR2 or mGluR5 corresponding to the intracellular pool (I)) or ~61kDa ($STEP_{61}$ isoform). Densities of the immunoreactive bands were analyzed using Lab Works (UVP, Upland, CA) or Un-Scan It (Silk Scientific, Orem, UT) software. Images were captured using a BioChemi Imaging System (UVP) coupled to a CCD camera or a scanner (Epson Electronics America, Inc., San Jose, CA) coupled to a PC computer. All samples were run at least twice and averaged across runs.

Data Summaries and Analyses

Behavior: For the acute treatment study, a Student's *t*-test was used to compare total session counts on Day 1 between saline and drug treatment. For the repeated treatment study, development of sensitization was determined using a one-way repeated measures analysis of variance (rmANOVA) comparing motor activity on Day1 and Day 3 between treatment groups with a *post hoc* Newman Keuls test for multiple comparisons. To determine expression of sensitization, a Student's *t*-test was used to compare motor responses between treatment groups. All data are presented as mean ± SEM. Significance was *a priori* set at $\alpha=0.05$

Immunoblots: The surface/intracellular ratio (S/I) of GluR1, GluR2, and mGluR5 was calculated by dividing the optical densities of the surface band by the intracellular band. Total GluR1, GluR2, and mGluR5 were determined by adding the optical densities of the surface and intracellular bands and dividing by the loading control actin. The portion of GluR1, GluR2, or mGluR5 on the surface was calculated by dividing the optical density of the surface band by actin; intracellular GluR1 or mGluR5 was similarly determined by dividing the optical density of the intracellular band by actin. Results from morphine-and Meth-treated rats were normalized to saline-treated (control) values for each brain region. All data are presented as mean + SEM. A Student's *t*-test was used to compare between saline control and drug treatment groups, with a Bonferroni correction for comparing the same control group to each of the two treatment groups, setting $\alpha=0.025$ for accepting significance.

Results

Behavioral Response to Acute Drug Administration

Rats treated with a single morphine injection (8mg/kg sc) demonstrated a significant decrease in horizontal activity ($t_{(14)}$=4.438, p<0.001) and repetitive beam breaks ($t_{(14)}$=4.682, p<0.001) compared to rats given saline (Table 2A). In contrast, a single injection of Meth (1mg/kg sc) resulted in a significant increase in horizontal activity (t_{14}=5.700, p<0.001) and vertical time ($t_{(14)}$=3.099, p<0.01) compared to saline treated rats (Table 2B).

Behavioral Response to Repeated Drug Administration

The behavioral responses to repeated drug administration are illustrated in Fig. 14 and 15. The development of morphine-induced sensitization was demonstrated by comparing motor activity on Days 1, 2 and 3. Horizontal beam breaks (Fig. 14A; $F_{(2,14)}$=7.94, p=0.005) and repetitive beam breaks (Figure 14B; $F_{(2,14)}$=6.49, p=0.009) were significantly increased with repeated morphine treatments. Similarly, motor sensitization developed following three injections of Meth. When motor activity was compared on Days 1, 2, and 3, vertical beam breaks (Fig. 15A; $F_{(2,14)}$=5.49, p=0.02) and vertical time (Figure 15B; $F_{(2,14)}$=4.67, p=0.03) were significantly increased. The sensitization was maintained for at least 14 days, for a between treatment group analysis showed that responding to an acute morphine challenge (8mg/kg sc; given on protocol Day 17) was greater in rats with a morphine history compared to those that received morphine for the first time (horizontal beam breaks: $t_{(13)}$=2.77, p=0.02; repetitive beam breaks:

$t_{(14)}$=2.76, p=0.02). When challenged with Meth 14 days later (Day 17), rats with a treatment history of Meth also had higher counts of activity compared to those with a saline treatment history (vertical beam breaks: $t_{(14)}$=2.45, p=0.03; vertical time: $t_{(14)}$=2.68, p=0.02), indicating that sensitization was maintained in the Meth-treated animals.

A separate group of animals was subjected to the same treatment protocols for Days 1-3, but on Day 17, brain tissue was harvested for biochemical analyses. As in the previous experiments, drug-treated rats demonstrated increased motor activity on Day 3 compared to Day 1. For morphine, horizontal beam breaks, $F_{(2,16)}$=13.68; repetitive beam breaks, $F_{(2,18)}$=11.35 (p<0.001).

For Meth, vertical beam breaks $F_{(2,18)}$=6.68, p=0.007; and vertical time, $F_{(2,16)}$=7.27, p=0.006. Thus, the biochemical results obtained on Day 17 for both morphine and Meth-treated rats represent neuronal adaptations that occur in the sensitized brain.

Effects of mGluR5 Negative Allosteric Modulator on development of motor sensitization

Miyatake has demonstrated that mGluR5 are involved in the development of Meth-induced CPP(Miyatake et al. 2005). We extended this literature by testing if these receptors were critical for the development of Meth-induced motor sensitization. As shown in Fig. 15, there was no effect of repeated vehicle and saline treatment (open triangles) or MTEP and Meth treatment (filled triangles) on vertical beam breaks ($F_{(1,17)}$=0.718, p=0.409) or vertical time

($F_{(1,16)}$=0.002, p=0.964). Vertical beam breaks did not show an effect of repeated treatment day ($F_{(1,34)}$=1.849, p=0.173) or an interaction between treatment and day of testing ($F_{(2,34)}$=2.042, p=0.145, Fig. 15C). Vertical time also did not shown an effect of repeated treatment day ($F_{(2,32)}$=1.33, p=0.278) nor was there a significant interaction between treatment and day of motor testing ($F_{(2,32)}$=1.01, p=0.376; Fig. 15D). These data indicate that development of motor sensitization did not occur after repeated treatment with Meth in rats pre-treated with the mGluR5 NAM, MTEP (5mg/kg, ip).

Effect of a single administration of morphine or Meth on the distribution of glutamate receptors.

There were minimal changes observed in the NAc (Table 3A&B) and VP (Table 3 A&B) one day after an acute morphine and Meth. Only the GluR1 S/I ratio was significantly decreased in the NAc of morphine compared to saline treated rats (Table 3A); yet, total, surface and intracellular GluR1 protein levels remained unchanged between treatment groups. There were no changes in S/I Ratio or total GluR2 or mGluR5 proteins in the NAc between saline and morphine treated rats (Table 3A). Furthermore, there were no changes in the S/I ratio or total GluR1, GluR2 or mGluR5 protein in the NAc between saline and Meth treated rats (Table 3B) or in the VP between saline and morphine (Table 4A) or saline and Meth treated rats (Table 4B).

Evaluations of immunoblots from tissue harvested on one day after morphine administration showed that the GluR1 S/I ratio was decreased in the

mPFC ($t_{(19)}$=2.853, p=0.0102). The decrease in the S/I ratio reflects the slight (but not significant) increase in the amount of intracellular GluR1 ($t_{(20)}$=1.996, p=0.0597), decrease in surface GluR1($t_{(20)}$=2.182, p=0.0412), and decrease in total GluR1 ($t_{(20)}$=2.187, p=0.0408) in morphine treated rats (Fig. 16B) (Note a Bonferroni correction was implemented for comparing the same control group to each of the two treatment groups, setting α=0.025 for accepting significance).

In the mPFC from morphine-treated rats, the amount of surface GluR2 was decreased ($t_{(18)}$=2.523, p=0.0213, Fig. 16D). However, there was not a significant change between saline and morphine treatment groups in total GluR2 (S+I, $t_{(18)}$=1.839, p=0.0825), S/I ratio ($t_{(18)}$=0.8660, p=0.3979) or intracellular protein ($t_{(18)}$=0.2862, p=0.7780). There was no change in the mPFC between saline and morphine treated rats in mGluR5 S/I ratio ($t_{(17)}$=1.476, p=0.1582), total protein (S+I, $t_{(17)}$=1.315, p=0.2059), surface protein ($t_{(17)}$=1.294, p=0.2130), or intracellular protein ($t_{(16)}$=1.423, p=0.1739, Fig. 16F). There also was no difference between saline and Meth treated rats in mPFC tissues with any glutamate receptor protein assayed (Table 5).

Effect of 14day withdrawal from repeated morphine or Meth administration on distribution of glutamate receptors.

No changes resulted in the NAc for GluR1, GluR2, or mGluR5 proteins following 14 days of withdrawal from repeated morphine (Table 6A) or Meth (Table 6B) treated rats (Student's *t*-test, p>0.025). Therefore, no $STEP_{61}$ protein levels were assessed in the NAc.

In VP tissue collected from rats treated with either morphine ($t_{(18)}$=2.653, p=0.016, Fig. 17B) or Meth ($t_{(19)}$=3.040, p=0.007, Fig. 17D), there was an increase in mGluR5 S/I ratio compared to the saline treatment group. This statistically significant increase in the mGluR5 S/I ratio reflected the non-significant trends in individual components: a slight increase in the surface component and a modest decrease in the intracellular mGluR5 pool. However, there was no change in any other mGluR5 protein component measured (Student's *t*-test, p>0.025, Fig. 17B and D). Moreover, no changes were detected in GluR1, GluR2 or $STEP_{61}$ proteins in the VP between saline and morphine (Table 7A) or Meth (Table 7B) (Student's *t*-test, p>0.025).

mPFC tissue collected 14 days after repeated morphine (8mg/kg), showed a significant increase in GluR2 S/I ratio of ($t_{(18)}$=2.997, p=0.008) compared to saline treated rats (Fig. 18C). Of note, is that the GluR2 I component demonstrated a non-significant trend towards a decrease ($t_{(19)}$=2.146, p=0.045) (Fig. 18C). There was no difference between treatment groups in the amount of GluR2 total protein ($t_{(19)}$=0.476, p=0.640) or S component ($t_{(19)}$=0.752, p=0.462) in mPFC tissues (Fig. 18C). Decreased in the amount of total mGluR5 ($t_{(19)}$=3.062, p=0.006) and S protein ($t_{(19)}$=2.691, p=0.014) were obtained in the mPFC of morphine-treated rats compared to saline controls (Fig. 18F). However, there was no change in the mGluR5 S/I ratio ($t_{(19)}$=2.057, p=0.054) or I protein ($t_{(19)}$=0.476, p=0.640) in this brain region (Fig. 18F). There was no change in any component of the GluR1 subunit of the AMPA receptor in the mPFC (Student's *t*-test, p>0.025, Fig. 18B) nor the $STEP_{61}$ protein (Fig. 18H).

Similar to what was observed subsequent to morphine treatment, after 14 days withdrawal from repeated Meth (1mg/kg), GluR2 S/I ratio was increased in the mPFC ($t_{(19)}$=3.475, p=0.002) (Fig. 19D). There was also a non-significant trend towards a decrease in the GluR2 I component of Meth-treated rats ($t_{(20)}$=2.385, p=0.027, Fig. 19D). However, there was no difference between treatment groups in total ($t_{(20)}$=0.086, p=0.932) or the S component ($t_{(20)}$=0.534, p=0.599) of GluR2 in the mPFC. Unlike what was detected after morphine treatment, there was a decrease in the level of $STEP_{61}$ protein in the mPFC of Meth- treated rats ($t_{(19)}$=2.504, p=0.022, Fig. 19H), but no change, in the protein components of mGluR5 (Fig. 19F) or GluR1 (Fig. 19B) (Student's *t*-test, p>0.025).

Effects of mGluR5 blockade on distribution of GluR2 and STEP61 subsequent to Meth withdrawal.

To determine if mGluR5 signaling was responsible for the changes in GluR2 surface expression and $STEP_{61}$ seen in the mPFC following repeated Meth, the mPFC was harvested after 14 days withdrawal from rats where the mGluR5 NAM, MTEP was shown to block the development of Meth-induced motor sensitization (motor data shown in Figure 15C and D). The changes that occurred subsequent to 14 days withdrawal from Meth in the mPFC were no longer present in GluR2 (Fig. 20B) and $STEP_{61}$ (Fig. 20D) of MTEP and Meth treated rats compared to vehicle and saline treated controls (Student's *t*-test, p>0.025).

Discussion

These studies demonstrate that administration of morphine (8mg/kg, sc) or Meth (1mg/kg, sc) produced an increased motor responding to a single injection. A progressive enhancement in motor behavior between the first and last of three, daily morphine or Meth administrations of morphine or Meth also occurred. Thus, harvesting tissue one day after the first injection, i.e., a time that equaled the inter-dosing interval used for the repeated injection, provided a temporal snapshot of the processes that occur during development of sensitization. The expression of motor sensitization was validated in a subset of rats behaviorally tested at fourteen days of withdrawal, suggesting that the brain was in a sensitized state at the time when tissue was harvested for biochemical analysis. We also determined that the mGluR5 NAM, MTEP (5mg/kg, ip), administered prior to Meth during development, precluded sensitization. This finding concurs with prior demonstration that mGluR5 is necessary for the development of Meth-induced conditioned place preference (Miyatake et al. 2005).

Several studies have reported changes in AMPA receptor and mGluR5 distribution in the NAc associated with cocaine treatment, a region important for the expression of sensitization. Cell surface and synaptic AMPA receptor levels are increased in the NAc after 2-3 weeks, but not one day of withdrawal from a sensitizing regimen of cocaine (Boudreau et al. 2007;Boudreau and Wolf 2005;Ghasemzadeh et al. 2009a). A single administration of amphetamine transiently decreases synaptosomal mGluR5 levels in the NAc one hour after

administration, an effect that is normalized five hours later (Shaffer et al. 2010). As with the AMPA receptors, synaptosomal mGluR5 levels remain unchanged one day after repeated cocaine administration; yet, these levels are also up-regulated following long-term (3 weeks) withdrawal in the NAc (Ghasemzadeh et al. 2009a). Supporting our negative findings in the NAc following repeated Meth treatment, Nelson and colleagues report that AMPA receptor S/I ratio is unchanged in the NAc of rats that are sensitized to amphetamine; however, acute amphetamine treatment slightly decreased surface levels of GluR1 after 24hr (Nelson et al. 2009). Therefore alterations in AMPA receptor subunits GluR1 and GluR2 appear to be unchanged following repeated Meth or amphetamine treatment in the NAc. Here we show that acute morphine treatment did not alter surface levels of GluR1; changes in the ratio were due to a non-significant increase in the intracellular levels of GluR1. Interpreted in the sense that surface AMPA receptors are the functional receptors, we would predict that AMPA receptor function in the NAc would not be affected by acute morphine treatment. This does not conflict with the role of NAc in the long-term effects of repeated drug exposure. AMPA receptors are clearly important for cue and drug-induced heroin seeking behavior at the withdrawal time examined in the current study (LaLumiere and Kalivas 2008); however, these receptors do not have to be up-regulated on the cell surface to produce these effects.

Our results indicate that specific up-regulation of mGluR5 S/I ratio is a common feature of withdrawal from a sensitizing regimen of both the opiate morphine and the stimulant Meth in the VP. Here we demonstrated that 14 days

after repeated morphine treatment, there was an increase in the mGluR5 S/I ratio due to a slight increase in the surface component of this receptor. Our lab has demonstrated that the VP is a critical mediator in the development (Johnson and Napier 2000;Mickiewicz et al. 2009), and maintenance (Dallimore et al. 2006;McDaid et al. 2006a) of opioid-induced motor sensitization. We have also shown that in VP neurons of morphine sensitized rats there is a significantly increased ability to enter depolarization block subsequent to local glutamate application (McDaid et al. 2006a). The activation of mGluR5 influences excitability in many ways, including the augmentation of ionotropic glutamate N-methyl-D-aspartic acid receptor (NMDAR) currents as well as suppression of after-hyperpolarization potassium currents (Ireland and Abraham 2002;Mannaioni et al. 2001). Therefore, it is possible that augmented mGluR5 S/I ratio could reduce recovery of VP neurons leading to increased excitability and depolarization inactivation in the presence of glutamate agonist. At this time point, however, we saw no alterations in the surface expression of GluR1 or GluR2 subsequent to morphine treatment in the VP. Alternative explanations for the observed increase in VP neuronal functionality include posttranslational modifications of AMPA receptors to alter the conductance of these channels (Derkach et al. 1999), changes in the cellular distribution of other AMPA receptor subunits (e.g. GluR3) or NMDA receptors. Electrophysiological studies with local application of selective agonists and antagonists for AMPA and NMDA receptors are needed to verify which receptor is functionally up-regulated. In the present study, we also observed an increase in the mGluR5 S/I ratio in the VP of rats 14

days after repeated Meth administration due to a decreased shift in the mGluR5 intracellular component. Therefore, mGluR5 levels are likely maintained on the cell surface while intracellular receptors are degraded, perhaps due to the lack of agonist-induced desensitization of mGluR5. VP neurons are also important for coding incentive motivational properties of drug cues following amphetamine sensitization (Tindell et al. 2005) and neuronal markers for activity as well as synaptogenesis in VP neurons are up-regulated following the expression of amphetamine-induced associative learning (Rademacher et al. 2006). Future electrophysiological assessments would help determine the role of mGluR5 on the excitability of VP neurons following withdrawal from repeated morphine and Meth.

We demonstrated here that acute morphine treatment significantly reduced the GluR1 S/I in the mPFC, and we propose this is a result of decreases in surface expression and increases in intracellular levels of GluR1, suggesting that GluR1 was redistributed from the surface to the intracellular compartment. These results are consistent with role of the mPFC in the development, but not expression, of morphine sensitization (Hao et al. 2007a;Tzschentke and Schmidt 1999). Long-term withdrawal from morphine treatment returned GluR1 levels to baseline. These results suggest that a decrease in responsiveness to excitatory inputs occurs within the inter-dosing interval timeframe of the treatments (24hr after acute or repeated treatment, Mickiewicz & Napier, *under review*) and prior to a long-term withdrawal period (14 days). In contrast to the observed effects following a single morphine treatment, there was no change in AMPA receptor

subunits in the mPFC after a single Meth injection. A recent study by Simoes and colleagues (Simoes et al. 2008) showed an increase in GluR2 in the frontal cortex 24hr after a single administration of meth. One possible explanation for the contrasting results was the dose of Meth, 30 mg/kg in the Simoes study compared to 1mg/kg used here.

At the long-term withdrawal period, both surface and total mGluR5 protein levels are decreased. Therefore, less mGluR5 was available to be activated in the mPFC of morphine-treated rats. Since mGluR5 activation results in internalization of AMPA receptor subunits (Snyder et al. 2001), it follows that the GluR2 S/I ratio was significantly increased after 14 days of withdrawal from repeated morphine. Though $STEP_{61}$ is a key mediator in mGluR5-dependent internalization of GluR2 (Zhang et al. 2008), this protein remained unchanged fourteen days after repeated morphine. However, the brain state of the mPFC following withdrawal from repeated morphine is adapting in such a way as to reduce neuronal calcium levels by favoring AMPA receptor subunit composition for the calcium-impermeable GluR2 (Liu and Zukin 2007) as well as decreasing mGluR5 surface and total protein which would reduce calcium release from intracellular stores (Sladeczek et al. 1985). Similarly, the GluR2 S/I ratio is increased following 14 days of withdrawal from repeated Meth due to a decreased shift in the intracellular GluR2 pool. No alterations occurred in mGluR5 levels in the mPFC. Since $STEP_{61}$ protein levels were significantly decreased, it appears that less phosphatase is available to internalize GluR2 receptors and levels increase on the membrane surface. We determined that this effect was mediated

through activation of mGluR5 receptors, as a pre-treatment of MTEP prior to Meth administration precluded any changes in mPFC GluR2 or STEP61 levels at 14 days of withdrawal. Therefore, the up-regulation of GluR2 S/I ratio and the decrease in STEP61 protein likely reflected activation of mGluR5 through Meth-induced increases in glutamate transmitter in the mPFC (Qi et al. 2009;Shoblock et al. 2003).

The current study characterized glutamate receptor adaptations induced by two drugs of separate classes: the opiate morphine and the stimulant Meth. Overlaps for the two drugs included the up-regulation of mGluR5 S/I ratio in the VP and GluR2 surface expression in the mPFC after long-term withdrawal. This suggests that there are common glutamatergic mechanisms between the adaptations that underlie sensitization.

Tables

Table 2A. Motor Activity evoked by a single injection of morphine.

	Saline	**Morphine**
Horizontal Activity	1306±50	555±126***
Repetitive Beam Breaks	822±46	339±74***

Table 1B. Motor activity evoked by a single injection of Methamphetamine.

	Saline	**Methamphetamine**
Vertical Activity	113±32	1924±385**
Vertical Time	70±20	651±143***

(A) Rats given a single administration of morphine (8mg/kg, sc, n=6) demonstrated a significant decrease in both horizontal activity and repetitive beam breaks compared to rats treated with saline (1ml/kg, sc, n=10). Behavioral assessments occurred over 90min post-injection. Student's *t*-test, ***p<0.001

(B) Rats given a single administration of Meth (1mg/kg sc; n=10) demonstrated a significant increase in both vertical activity and vertical time compared to rats treated with saline (1ml/kg sc; n=6). Behavioral assessments occurred over 60min post-injection. Student's *t*-test, **p<0.01,***p<0.001

Table 3A. NAc: 1 Day After Morphine.

Protein:	Component	Saline	Morphine	Statistics
GluR1	S/I	103.1±10.6	68.3±7.3	$t_{(18)}$=2.581, p=0.019*
	Total	100.3±6.9	102.2±4.0	$t_{(19)}$=0.238, p=0.814
GluR2	S/I	97.6±5.2	113.7±10.7	$t_{(17)}$=1.394, p=0.181
	Total	115.9±7.6	112.5±6.6	$t_{(18)}$=0.335, p=0.741
mGluR5	S/I	100.0±7.8	101.7±16.0	$t_{(18)}$=0.103, p=0.919
	Total	98.7±10.2	114.8±11.3	$t_{(19)}$=1.061, p=0.302

Table 3B. NAc: 1 Day After Methamphetamine.

Protein:	Component	Saline	Meth-amphetamine	Statistics
GluR1	S/I	103.1±10.6	142.4±28.3	$t_{(18)}$=1.405, p=0.177
	Total	100.3±6.9	112.1±6.4	$t_{(19)}$=1.251, p=0.226
GluR2	S/I	97.6±5.2	97.3±4.5	$t_{(18)}$=0.047, p=0.963
	Total	115.9±7.6	108.1±12.7	$t_{(18)}$=0.550, p=0.589
mGluR5	S/I	100.0±7.8	103.5±12.1	$t_{(20)}$=0.246, p=0.808
	Total	98.7±10.2	106.5±8.8	$t_{(20)}$=0.577, p=0.570

Shown are the mean ± SEM optical density values as percent average saline control for each treatment group and corresponding Student's *t*-test statistics. S/I ratio and Total (S+I) protein were assessed for GluR1, GluR2, and mGluR5 in

VP tissues after one day of a single injection of Saline (1ml/kg, sc), **(A)** morphine (8mg/kg, sc) or **(B)** Meth(1mg/kg, sc). There was a significant decrease in the GluR1 S/I ratio in the NAc of morphine-treated rats *p<0.025. However, there were no other differences between saline and Meth treatment groups for any glutamate receptor protein component assayed. Shown are mean ± SEM optical density values as percent average saline control for each treatment group and corresponding Student's *t*-test statistics, with Bonferroni correction as saline groups were used for morphine and Meth comparisons, α=0.025.

Table 4A. VP: 1 Day After Morphine.

Protein:	Component	Saline	Morphine	Statistics
GluR1	S/I	100.9±12.2	133.6±21.8	$t_{(20)}$=1.370, p=0.186
	Total	99.6±5.7	111.5±7.0	$t_{(20)}$=1.328, p=0.199
GluR2	S/I	100.0±7.1	115.0±8.8	$t_{(18)}$=1.340, p=0.197
	Total	100.0±4.5	96.0±3.9	$t_{(18)}$=0.649, p=0.525
mGluR5	S/I	100.0±9.7	131.4±22.1	$t_{(20)}$=1.381, p=0.182
	Total	95.6±5.14	118.2±12.5	$t_{(19)}$=1.729, p=0.100

Table 4B. VP: 1 Day After Methamphetamine.

Protein:	Component	Saline	Meth-amphetamine	Statistics
GluR1	S/I	100.9±12.2	108.7±16.0	$t_{(20)}$=0.397, p=0.696
	Total	99.6±5.7	101.9±6.3	$t_{(20)}$=0.279, p=0.783
GluR2	S/I	100.0±7.1	105.3±11.4	$t_{(18)}$=0.412, p=0.685
	Total	100.0±4.5	98.3±6.2	$t_{(18)}$=0.222, p=0.827
mGluR5	S/I	100.0±9.7	97.8±14.6	$t_{(19)}$=0.129, p=0.898
	Total	95.6±5.1	135.1±18.3	$t_{(19)}$=2.164, p=0.043

S/I ratio and Total (S+I) protein were assessed for GluR1, GluR2, and mGluR5 in the VP tissues after one day of acute Saline (1ml/kg, sc), **(A)** morphine (8mg/kg, sc) or **(B)** Meth (1mg/kg, sc) treatment. There were no differences between saline and morphine treatment groups for any glutamate receptor protein component assayed. Shown are mean ± SEM optical density values as percent

average saline control for each treatment group and corresponding Student's *t*-test statistics, $\alpha=0.025$.

Table 5. mPFC: 1 Day After Methamphetamine.

Protein:	Component	Saline	Meth-amphetamine	Statistics
GluR1	S/I	100.9±17.2	81.5±19.6	$t_{(18)}$=0.733, p=0.473
	Total	100.7±3.7	92.4±4.9	$t_{(19)}$=1.376, p=0.185
GluR2	S/I	100.4±9.7	95.4±14.3	$t_{(17)}$=0.296, p=0.770
	Total	101.5±6.7	88.6±5.8	$t_{(17)}$=1.285, p=0.216
mGluR5	S/I	101.5±10.3	136.2±27.6	$t_{(16)}$=1.281, p=0.218
	Total	100.1±6.7	100.2±8.4	$t_{(16)}$=0.008, p=0.994

S/I ratio and Total (S+I) protein were assessed for GluR1, GluR2, and mGluR5 in the mPFC tissues after one day of acute Saline (1ml/kg, sc) or Meth (1mg/kg, sc) treatment. There was no difference between treatment groups for any glutamate receptor protein assayed. Shown are mean ± SEM optical density values as percent average saline control for each treatment group and corresponding Student's *t*-test statistics, α=0.025 (Bonferroni correction).

Table 6A. NAc: 14 Days After Morphine.

Protein:	Component	Saline	Morphine	Statistics
GluR1	S/I	99.9±7.5	154.2±32.9	$t_{(19)}$=1.844, p=0.081
	Total	106.4±7.2	111.1±7.3	$t_{(20)}$=0.447, p=0.659
GluR2	S/I	99.6±9.0	113.6±16.6	$t_{(19)}$=0.762, p=0.455
	Total	99.5±4.5	91.2±8.8	$t_{(19)}$=0.855, p=0.403
mGluR5	S/I	101.3±11.7	129.2±10.8	$t_{(20)}$=1.726, p=0.099
	Total	100.9±3.6	101.5±6.3	$t_{(20)}$=0.079, p=0.938

Table 6B. NAc: 14 Days After Methamphetamine.

Protein:	Component	Saline	Meth-amphetamine	Statistics
GluR1	S/I	99.9±7.5	84.1±10.2	$t_{(19)}$=1.278, p=0.217
	Total	106.4±7.2	113.7±7.1	$t_{(20)}$=0.716, p=0.482
GluR2	S/I	99.6±9.0	101.2±13.5	$t_{(18)}$=0.102, p=0.920
	Total	99.5±4.5	84.9±9.4	$t_{(17)}$=1.524, p=0.146
mGluR5	S/I	101.3±11.7	118.5±23.7	$t_{(19)}$=0.706, p=0.489
	Total	100.9±3.6	94.0±5.1	$t_{(20)}$=1.141, p=0.267

There were no differences between **(A)** saline and morphine (8mg/kg sc, once daily for 3 days) or **(B)** saline (1ml/kg sc, once daily for 3 days) and Meth treatment (1mg/kg sc for 3 days) groups for any glutamate receptor protein

component assayed. Shown are the mean ± SEM optical density values as percent average saline control for each treatment group and corresponding Student's *t*-test statistics, α=0.025 (Bonferroni correction).

Table 7A. VP: 14 Days After Morphine.

Protein:	Component	Saline	Morphine	Statistics
GluR1	S/I	99.1±9.0	123.1±23.4	$t_{(19)}$=1.057, p=0.304
	Total	99.7±5.6	99.4±8.6	$t_{(20)}$=0.037, p=0.970
GluR2	S/I	98.9±6.3	99.6±11.0	$t_{(20)}$=0.058, p=0.954
	Total	101.1±5.7	102.0±10.3	$t_{(20)}$=0.075, p=0.941
$STEP_{61}$		100.0±8.3	94.1±8.2	$t_{(20)}$=0.503, p=0.621

Table 7B. VP: 14 Days After Methamphetamine.

Protein:	Component	Saline	Meth-amphetamine	Statistics
GluR1	S/I	99.1±9.0	114.5±15.2	$t_{(20)}$=0.906, p=0.376
	Total	100.6±5.8	109.6±3.6	$t_{(20)}$=1.258, p=0.223
GluR2	S/I	98.9±6.3	88.8±13.4	$t_{(19)}$=0.735, p=0.471
	Total	101.1±5.7	130.0±12.6	$t_{(20)}$=2.262, p=0.035
$STEP_{61}$		100.0±8.3	107.3±9.6	$t_{(19)}$=0.573, p=0.574

There were no differences between **(A)** saline and morphine (8mg/kg sc, once daily for 3 days) or **(B)** saline (1ml/kg sc, once daily for 3 days) and Meth treatment (1mg/kg sc for 3 days) groups for any glutamate receptor protein component assayed. Shown are the mean ± SEM optical density values as

percent average saline control for each treatment group and corresponding Student's *t*-test statistics, α=0.025 (Bonferroni correction).

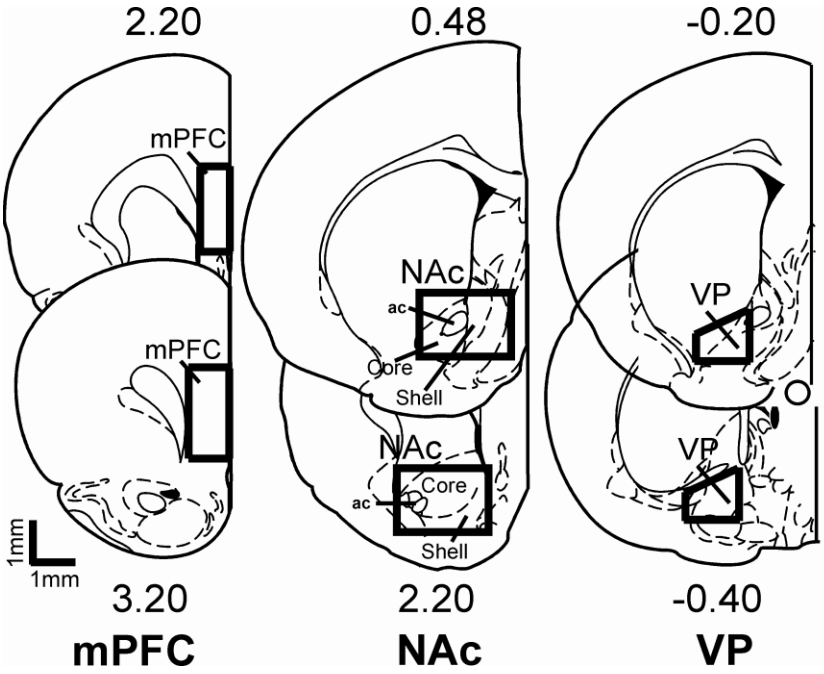

Figure 13. Stereotaxic maps of brain regions assayed. Illustrations of stereotaxic maps of rodent brain were modified from Paxinos and Watson (1998). The bold outlines correspond to dissections of the medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and ventral pallidum (VP) brain tissues assayed in the current study. Numbers indicate distance in millimeters of the respective brain section from bregma.

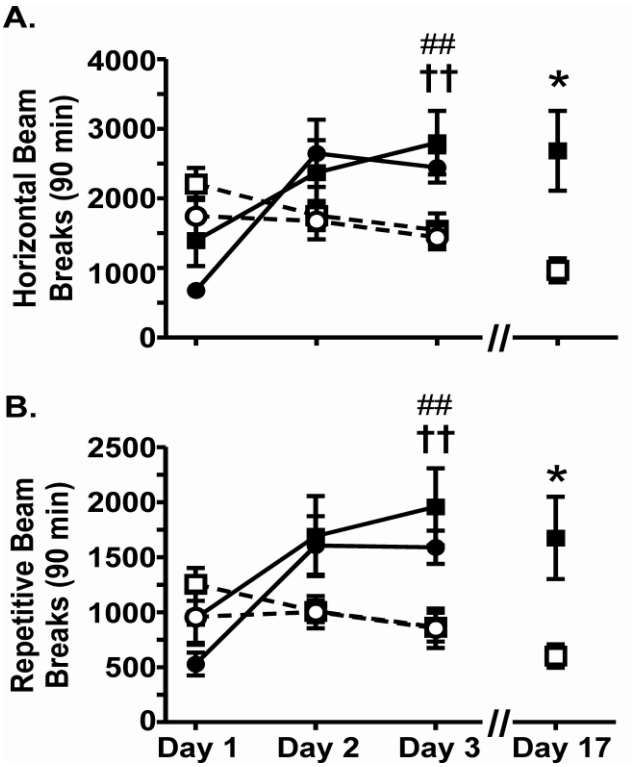

Figure 14. Development and expression of morphine sensitization. Data were collapsed for 90min after administration of morphine. **A)** Horizontal Beam Breaks, **B)** Repetitive beam breaks. There was no statistical difference between days 1, 2 or 3 of the saline treatment (open symbols, n=6-8). In the morphine-treated groups, there were significant differences between treatment days (filled circles=animals used for biochemistry; filled squares=animals used for behavioral verification). A challenge injection of morphine was administered on day 17 (14 days withdrawal) to rats with a saline (open square, n=7-8) or morphine (filled square, n=8) treatment history; rats with a morphine treatment history expressed sensitization. One-way rmANOVA with *post hoc* Newman-Keuls, ## $p<0.01$ (biochemistry group), †† $p<0.01$ (behavior group); Student's *t*-test, * $p<0.05$.

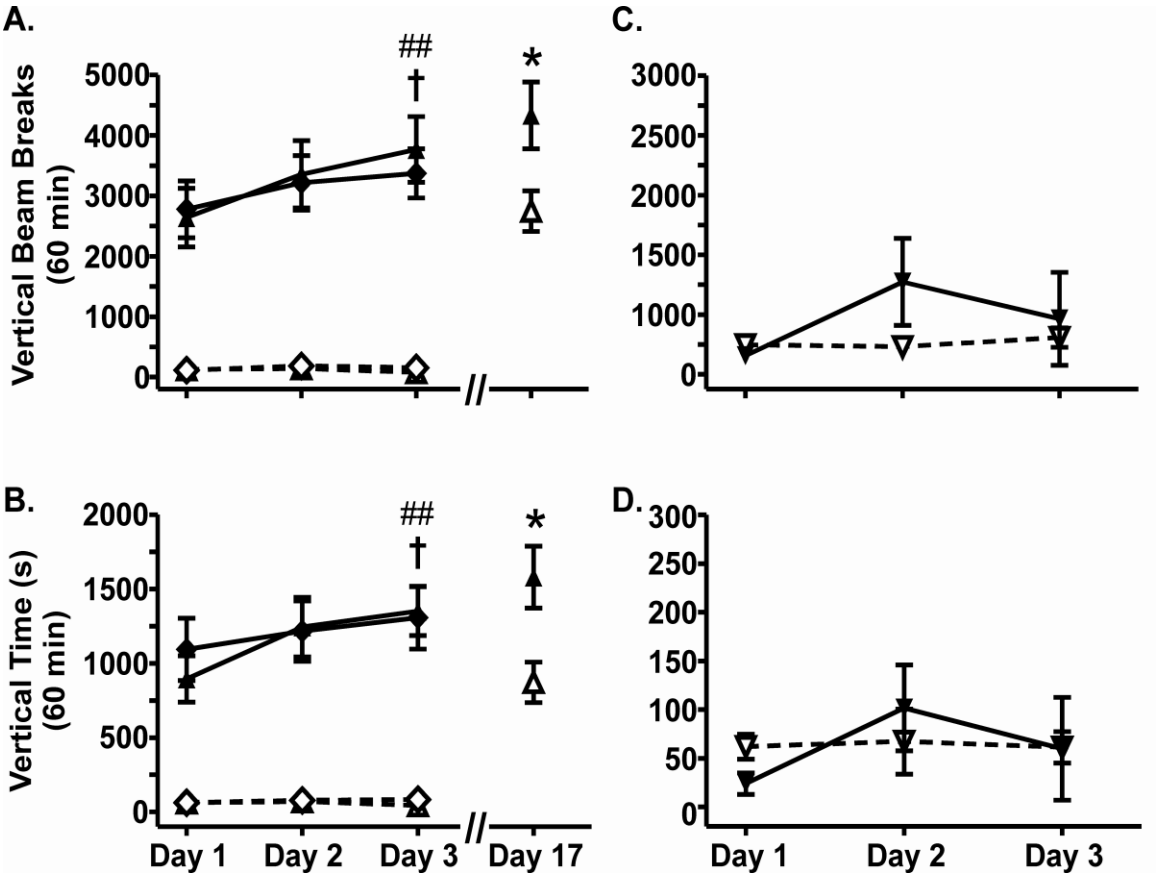

Figure 15. Development and expression of Meth sensitization. Data were collapsed for 60min after administration of Meth. **A)** Vertical Beam Breaks, **B)** Vertical Time. There was no statistical difference between days 1, 2 or 3 of the saline treatment (open symbols, n=6-8). In the Meth-treated groups, there were significant differences between treatment days (filled diamonds = animals used for biochemistry; filled triangles = animals used for behavioral verification). A challenge injection of Meth was administered on day 17 (14 days withdrawal) to rats with a saline (open triangle, n=8) or Meth (filled triangle, n=8) treatment history; rats with a Meth treatment history expressed sensitization. There was no

development of motor sensitization from Day 1 to Day 3 assessed by Vertical Beam Breaks **C)**, or Vertical Time **D),** in rats treated with MTEP and Meth (solid triangles) or Vehicle and saline (open triangles). There was also no difference between treatment groups in either motor activity parameter on Day 1 of drug administration. One-way rmANOVA with *post hoc* Newman-Keuls, ## $p<0.01$ (biochemistry group), † $p<0.05$ (behavior group); Student's *t*-test, * $p<0.05$.

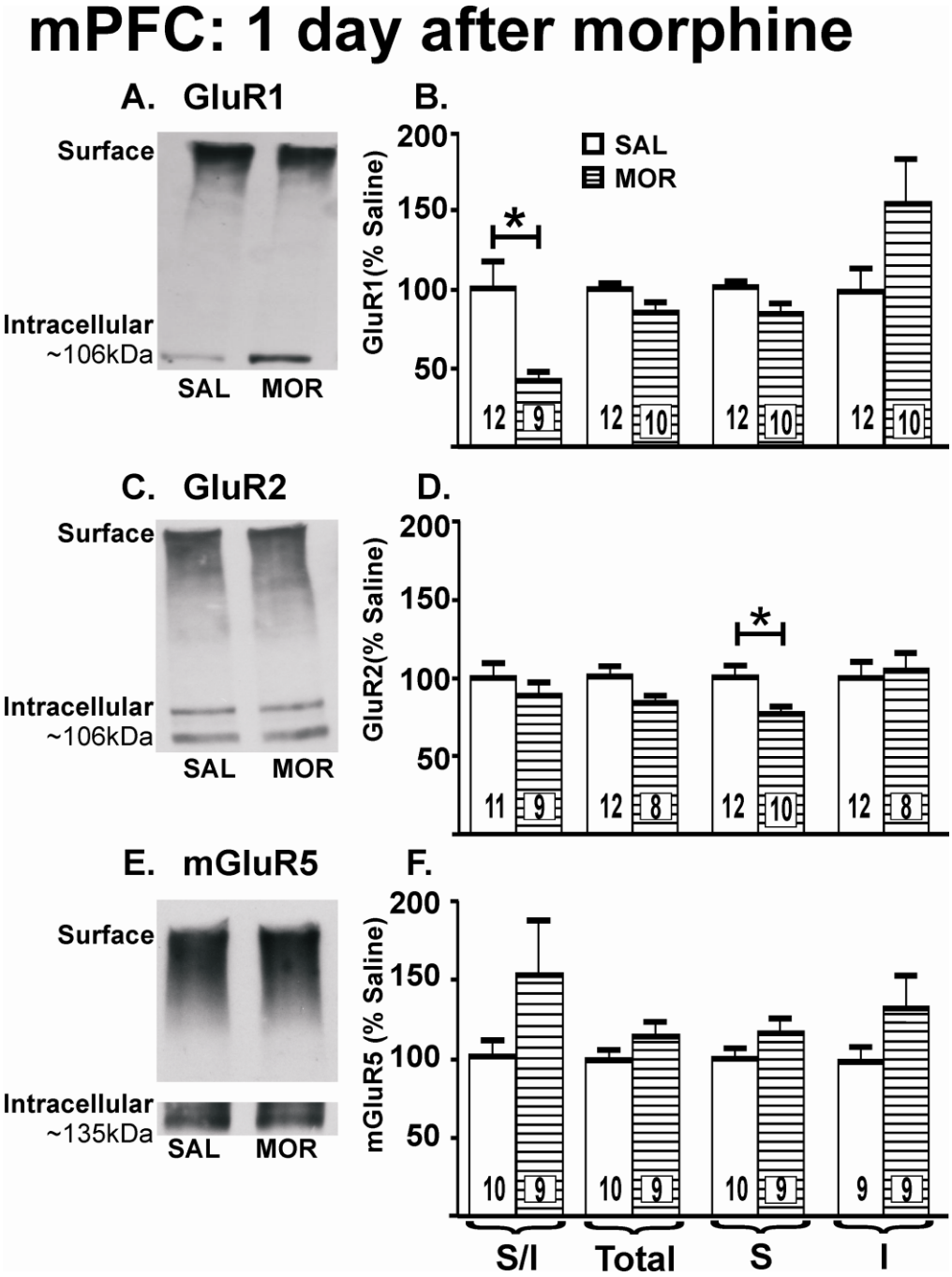

Figure 16. Decreased surface expression of GluR1 and GluR2 occurred in the mPFC of rats treated acutely with morphine.

Representative immunoblots for **A.** GluR1, **C.** GluR2, and **E.** mGluR5 in mPFC tissues harvested one day after a single injection of saline (SAL, 1ml/kg, sc) or

morphine (MOR, 8mg/kg, sc) treatment. Distinct surface and intracellular components are labeled for each protein on each representative immunoblot with corresponding molecular weight of the intracellular component quantified. **B.** GluR1 S/I ratio was decreased in MOR treated rats, but Total, S and I components remain unchanged. **D.** GluR2 S component was decreased in MOR treated rats but S/I ratio, Total and I GluR2 protein did not change. **F.** mGluR5 S/I ratio, Total, S and I protein did not change between SAL and MOR treated rats in the mPFC. White bars represent saline (SAL) and white bars with horizontal lines represent morphine (MOR) treated rats. Numbers within bars represent sample number (N). Unpaired *t*-test, *$p<0.025$.

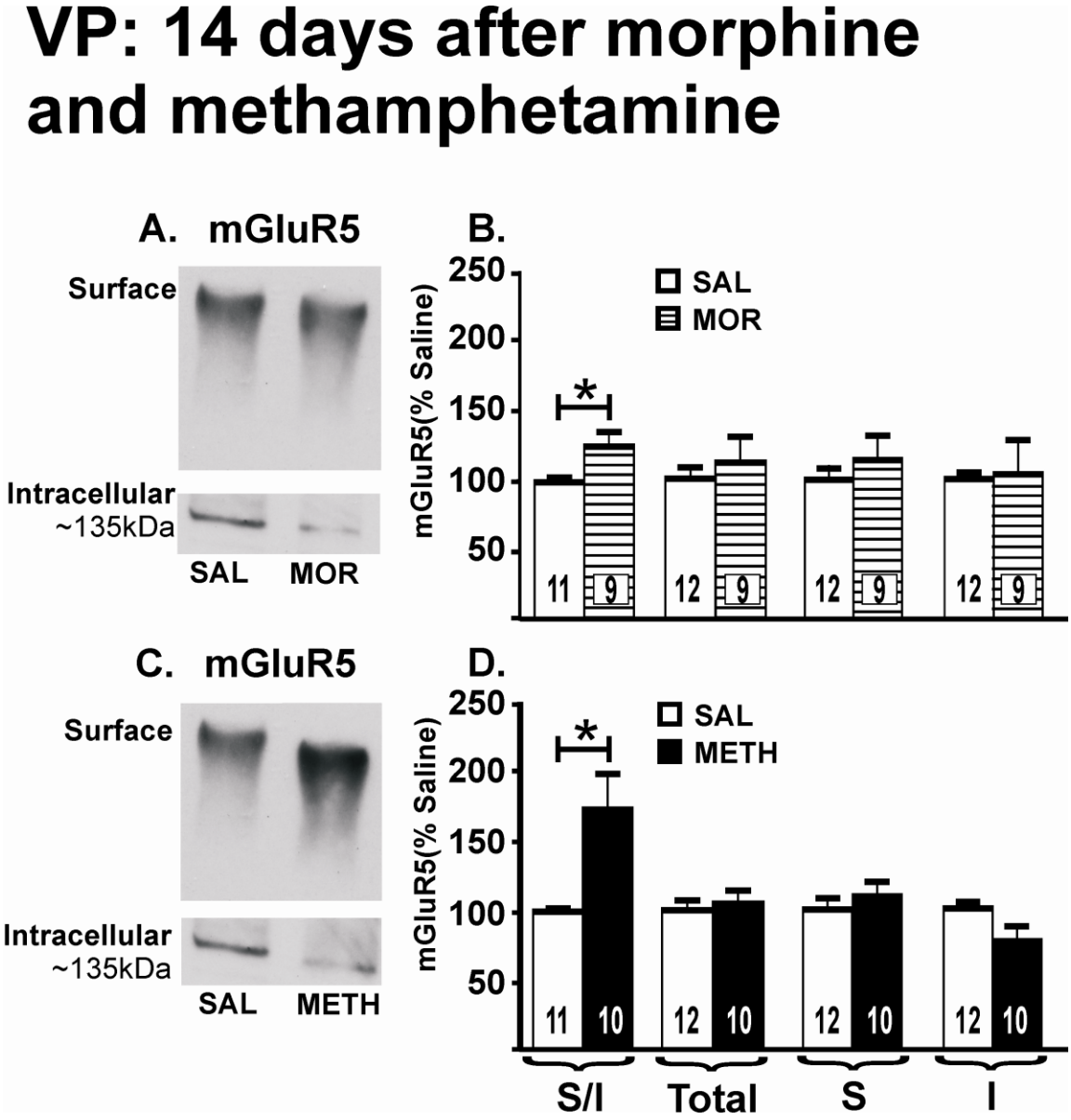

Figure 17. Increased mGluR5 surface expression occurred in the VP 14 days after morphine or Meth treatment. Representative immunoblots probed for mGluR5 in the VP 14 days after morphine (MOR, 8mg/kg, sc) **A.** or Meth (METH, 1mg/kg, sc) **C.** treatment. Distinct surface and intracellular components are labeled for each protein on each representative immunoblot with corresponding molecular weight of the intracellular component quantified. **B.**

The mGluR5 S/I ratio was increased in MOR treated rats but Total, S and I components remained unchanged. **D.** The mGluR5 S/I ratio was also increased in the VP of METH compared to saline treated rats. However, mGluR5 Total, S and I protein components were not altered by treatment history. White bars represent saline (SAL), white bars with horizontal lines represent morphine (MOR), and filled black bars represent Meth (METH) treated rats. Numbers within bars represent sample number (N). Student's *t*-test, *p<0.025.

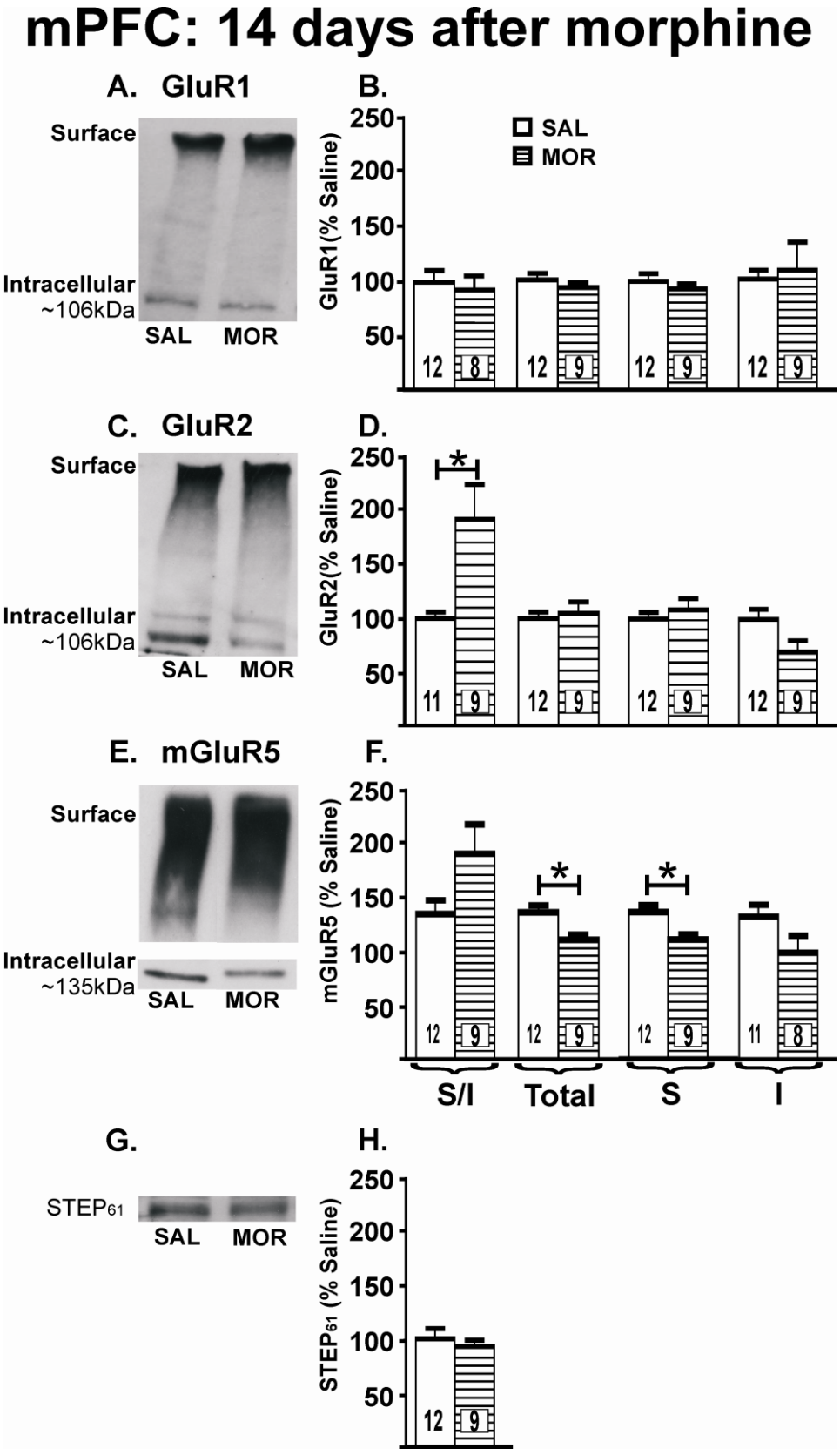
mPFC: 14 days after morphine
A. GluR1
Surface
Intracellular
~106kDa
SAL
MOR
B.
SAL
MOR
GluR1(% Saline)
250
200
150
100
50
12
8
12
9
12
9
12
9
C. GluR2
Surface
Intracellular
~106kDa
SAL
MOR
D.
GluR2(% Saline)
*
11
9
12
9
12
9
12
9
E. mGluR5
Surface
Intracellular
~135kDa
SAL
MOR
F.
mGluR5 (% Saline)
*
*
12
9
12
9
12
9
11
8
S/I
Total
S
I
G.
STEP61
SAL
MOR
H.
STEP61 (% Saline)
12
9

Figure 18. Surface expression of GluR2 is increased in the mPFC 14 days after morphine administration. Representative immunoblots for **A.** GluR1, **C.** GluR2, **E.** mGluR5, and **G.** $STEP_{61}$ in mPFC tissues harvested 14 days after repeated injections of saline (SAL, 1ml/kg, sc) or morphine (MOR, 8mg/kg, sc) treatment. **B.** The GluR1 S/I ratio, Total, S and I protein components remained unchanged between saline and morphine treatment groups in the mPFC 14 days after morphine administration. **C.** There was a significant increase in the GluR2 S/I ratio of morphine compared to saline treated rats in the mPFC without a change in Total, S or I GluR2 protein components. **F.** There was a significant decrease in mGluR5 Total and S components of morphine treated rats without a change in the mGluR5 S/I ratio or I component between saline and morphine treatment groups. **H.** There was no difference between saline and morphine treatment groups in the level of $STEP_{61}$ after 14 days in the mPFC. White bars represent saline (SAL) and white bars with horizontal lines represent morphine (MOR) treated rats. Numbers within bars represent sample number (N). Unpaired *t*-test, *$p<0.025$.

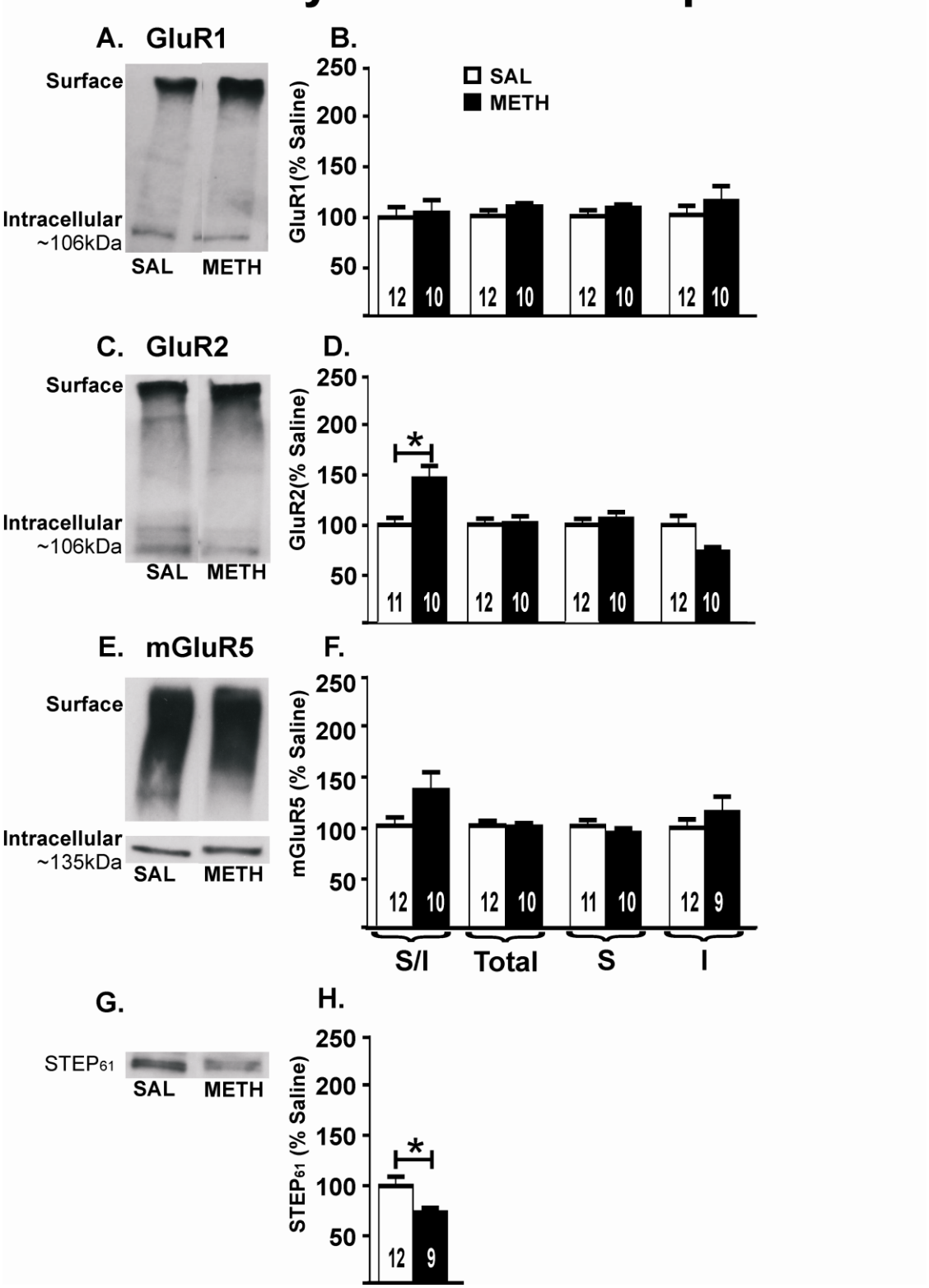
mPFC: 14 days after methamphetamine
A. GluR1
Surface
Intracellular
~106kDa
SAL
METH
B.
SAL
METH
GluR1(% Saline)
250
200
150
100
50
12
10
C. GluR2
Surface
Intracellular
~106kDa
SAL
METH
D.
GluR2(% Saline)
*
11
E. mGluR5
Surface
Intracellular
~135kDa
SAL
METH
F.
mGluR5 (% Saline)
9
S/I
Total
S
I
G.
STEP61
SAL
METH
H.
STEP61 (% Saline)

Figure 19. GluR2 surface expression is increased and $STEP_{61}$ levels were decreased 14 days after repeated Meth administration in the mPFC. Representative immunoblots for **A.** GluR1, **C.** GluR2, **E.** mGluR5, and **G.** $STEP_{61}$ in mPFC tissues harvested 14 days after repeated injections of saline (SAL, 1ml/kg, sc) or Meth (METH, 1mg/kg, sc) treatment. **B.** There was no difference in GluR1 S/I ratio, Total, S or I components in the mPFC between rats with a saline or Meth treatment history. **D.** The GluR2 S/I ratio was elevated in Meth compared to saline treated rats 14 days after drug administration. However, GluR2 Total, S and I components remained unchanged. **E.** mGluR5 S/I ratio, Total, S and I components remain unchanged 14 days after saline and Meth in mPFC tissue. **H. STEP61** was significantly reduced in the mPFC of rats 14 days after Meth compared to saline. White bars represent saline (SAL) and filled black bars represent Meth (METH) treated rats. Numbers within bars represent sample number (N). Student's *t*-test, *$p<0.025$.

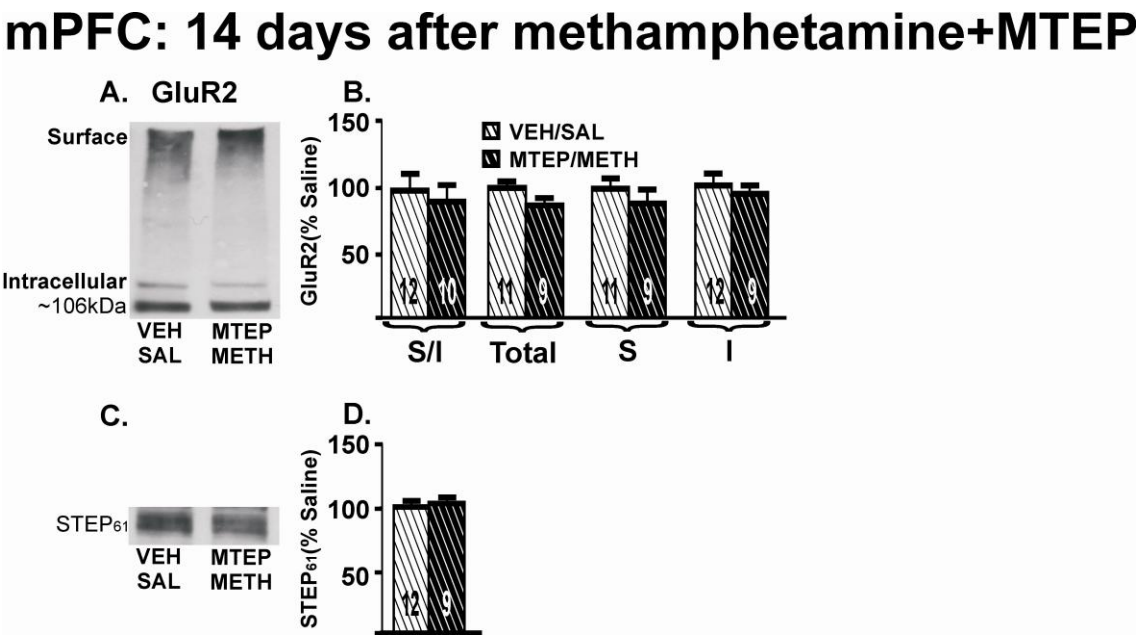

Figure 20. Levels of GluR2 and $STEP_{61}$ remain unaltered 14 days following repeated treatment with mGluR5 antagonist, MTEP, and Meth in the mPFC. Representative immunoblots for **A.** GluR2 and **C.** $STEP_{61}$ in mPFC tissues harvested 14 days after a repeated injections of vehicle and saline (VEH/SAL; 1ml/kg, ip; 1ml/kg sc) or MTEP and Meth (MTEP/METH; 5mg/kg, ip; 1mg/kg, sc) treatment. **B.** There are no GluR2 S/I ratio, Total protein, S or I protein component differences between VEH/SAL and MTEP/METH treated rats in mPFC. **D.** There are no differences in $STEP_{61}$ levels between VEH/SAL and MTEP/METH treated rats in the mPFC. White bars with slanted black lines represent vehicle and saline treated rats (VEH/SAL) and filled black bars with slanted white lines represent MTEP and Meth (MTEP/METH) treated rats. Numbers within bars represent sample number (N). Student's *t*-test, $p>0.025$.

CHAPTER VII

PHARMACOLOGICAL AND DEVELOPMENTAL MODELS OF SCHIZOPHRENIA: DIFFERENCES IN SENSORIMOTOR GATING, AND RESPONDING TO METHAMPHETAMINE AND AN mGluR5 POSITIVE ALLOSTERIC MODULATOR

Abstract

Schizophrenia patients have a higher incidence of substance use disorders than the general population. Amphetamines including methamphetamine (Meth) exacerbate symptoms of psychosis in schizophrenia patients. Our objective was to study the detrimental effects of stimulants on deficits associated with schizophrenia and the underlying sensitivity of this population to stimulant addiction. We chose to induce a schizophrenia-like brain state *via* pharmacological and developmental means with two well-established models of this neuropathology: repeated, escalating amphetamine (Amph) (Peleg-Raibstein et al. 2008) and isolation rearing (Geyer et al. 1993). Both of these models are known to produce deficits in sensorimotor gating assessed by prepulse inhibition (PPI) of the acoustic startle response. Therefore, we measured PPI deficits in both models and compared these assessments with the rewarding and motor responses to Meth *via* conditioned place preference (CPP) and motor sensitization, respectively.

CPP implements reward and associative learning, both of which are associated with negative symptoms of schizophrenia; thus, we hypothesized that sensorimotor gating deficits would be inversely correlated with Meth-induced CPP. Because motor sensitization in rodents is hypothesized to parallel the course of psychosis in humans and positive symptoms of schizophrenia, we hypothesized that PPI deficits would be positively correlated with Meth-induced activity and motor sensitization. We found that PPI deficits in both pharmacological and developmental schizophrenia models negatively correlated with Meth-induced CPP. Both schizophrenia-like rodent models also demonstrated enhanced sensitivity to the hypermotoric effects of Meth. By strengthening the signal of associative learning with the addition of the Meth cue or by enhancing the glutamatergic system, Meth-induced CPP was enhanced. These novel findings demonstrate that schizophrenia-like rodents are less sensitive to the rewarding and more sensitive to the hypermotoric properties of Meth. Sensorimotor gating deficits can predict both of these Meth-induced behaviors. Therefore, this work provides pre-clinical rationale for the use of PPI as a predictor for Meth addiction in schizophrenia patients.

Introduction

A diagnosis of schizophrenia increases the likelihood of a substance abuse disorder by approximately five times over that of the general United States population (Regier et al. 1990). A recent clinical assessment demonstrated that 25% of schizophrenia patients also meet criteria for psychostimulant dependence

(Compton et al. 2005). This dual diagnosis is particularly problematic for stimulants because these abused drugs exacerbate symptoms in schizophrenia patients (Angrist et al. 1980;Janowsky and Davis 1976). Methamphetamine (Meth) is a powerful stimulant with high abuse liability and remains the third most widely used illicit drug world-wide (United Nations Office on Drugs and crime 2009). It is well documented the Meth abuse can induce a psychotic state similar to that of schizophrenia (Harris and Batki 2000;McKetin et al. 2006;Scott et al. 2007). Therefore, elucidating correlations between behaviors associated with the schizophrenia brain state and Meth-abuse would provide insight into each disorder as well as the dual diagnosis.

Behavioral overlaps between laboratory animals and humans provide a powerful means to model, and thus study, the human condition. Sensorimotor gating deficits measured by prepulse inhibition (PPI) occur in human schizophrenia patients and in rodent models of this disorder, illustrating the cognitive fragmentation that occurs with this neuropathology (Braff et al. 2001;Swerdlow and Geyer 1998). Acoustic startle response PPI refers to a normal suppression of the startle reflex that occurs when a strong startling stimulus is preceded by a weaker stimulus (i.e., a warning signal, termed the 'pre-pulse') (Graham 1975;Hoffman and Searle 1968;Ison et al. 1973;Swerdlow and Geyer 1998). Deficits in this behavior can be induced by pharmacological or non-pharmacological means. Acute (Swerdlow et al. 2003) and repeated (Peleg-Raibstein et al. 2008) amphetamine administration induce PPI deficits in the acoustic startle response in rodents and humans. These deficits are reversed by

antipsychotic drugs (Geyer et al. 2001), which supports the value of this measure as an indicator of the schizophrenia-like brain. Early environmental stressors, such as rearing young rats in isolation, result in sensorimotor gating deficits that are also reversed by antipsychotic medication (Bakshi et al. 1998;Geyer et al. 1993). Both Amph treatment and isolation rearing in laboratory rats are widely used to model aspect of the human schizophrenia brain. Since these two models are produced by vastly different means, with differential effects on the adult brain, we sought to compare them in terms of modeling the co-morbidity of schizophrenia and stimulant abuse.

Human stimulant abuse is modeled in rodents via a wide variety of paradigms and outcome measures. Key to the phenomenon of drug addiction is the neuronal adaptations that are imposed by repeated drug exposure. Behavioral outcomes of such adaptations that are easily measured in laboratory rats include stimulant-induced motor activity and associative learning. The augmentation of motoric responses to stimulants that occurs after repeated treatment is termed motor sensitization (Stewart and Badiani 1993). The acute effects of Amph (e.g., euphoria, hyper-vigilance) appear to “sensitize” with repeated exposure and can result in psychotic state that is akin to the positive symptoms of schizophrenia (Angrist et al. 1980;Strakowski et al. 1996). Developmental rodent models of schizophrenia, including isolation rearing, render rodents more sensitive to the motor stimulating effects of cocaine, nicotine, Amph, and Meth (Berg and Chambers 2008;Chambers and Taylor 2004;Dai et al. 2004;Smith et al. 1997). Thus, motor sensitization appears to be

an excellent outcome measure that mirrors the clinical evidence for an increased propensity for substance abuse in schizophrenia. Self-administration of cocaine and Meth are also enhanced in developmental, rodent models of schizophrenia, indicating that these drugs are more reinforcing in a schizophrenia brain state (Brady et al. 2008;Chambers and Self 2002). Associative learning underlies the well-described phenomenon wherein environmental cues associated with drug administration take on the salience of the drug, and exposure to these cues can evoke drug craving in drug-withdrawn addicts (Hartz et al. 2001;Hogarth et al. 2010;Mucha et al. 1998;Panlilio et al. 2005;Tolliver et al. 2010). Conditioned place preference (CPP) is a classical conditioning paradigm that can assess salience attributed to drug-associated contextual cues in both rodents and humans (Childs and deWit H. 2009;Tzschentke 1998;Tzschentke 2007). In the CPP paradigm, unique contextual cues (the conditioned stimulus) are temporally paired with a reward (e.g., a stimulant; the unconditioned stimulus), and once an association is formed between the context and the stimulant, the subject tends to spend more time in that context (or place) even in a drug-free state. The CPP procedure involves components of reward as well as associative learning, both of which are deficits associated with the negative symptoms of schizophrenia (i.e., anhedonia and working memory deficits). Schizophrenia-like rodents fail to demonstrate drug-induced CPP subsequent to conditioning with morphine or amphetamine (Le et al. 2002;Wongwitdecha and Marsden 1995;Wongwitdecha and Marsden 1996). These studies demonstrate that CPP paradigms serve as a useful index of negative symptoms of schizophrenia such as anhedonia and

working memory deficits, whereas motor sensitization may be more analogous to the positive symptoms. The objective for the current study was to compare behavioral outcomes associated with schizophrenia in both pharmacological and developmental models of the disorder and to correlate these outcomes with Meth-induced CPP and motor sensitization in the same animal. We hypothesized that sensorimotor gating deficits would be inversely related to Meth-induced CPP and positively related with motor sensitization.

The brain state that is reflected schizophrenia and stimulant addiction includes a dysregulation in glutamate transmission (Bardo 1998;Carlsson et al. 1999;Goff and Coyle 2001;Tzschentke 1998). One regulator of glutamate transmission is the group I subtype 5 metabotropic glutamate receptor (mGluR5). This receptor is highly expressed in brain regions important for stimulant reward and associative learning including the nucleus accumbens, medial prefrontal cortex and hippocampus (Kerner et al. 1997;Lu et al. 1999;Testa et al. 1994b). The mGluR5 is critical for the acquisition of Meth-induced CPP (Miyatake et al. 2005), expression of Amph-induced CPP (Herzig et al. 2005), as well as Meth self-administration (Osborne and Olive 2008) and the cue-induced reinstatement of this behavior (Gass et al. 2009). Alternatively, augmenting mGluR5 activity through the use of a selective positive allosteric modulator (PAM) facilitates extinction learning of cocaine-induced CPP (Gass and Olive 2009). Schizophrenia-like rats demonstrate that Meth is, in fact, reinforcing in self-administration paradigms. Thus, by enhancing the glutamatergic signal during the acquisition or expression process of CPP,

schizophrenia-like rodents may be better able to make Meth-induced associations. We therefore hypothesized that administration of an mGluR5 PAM during conditioning would enhance Meth-induced associative learning in rodent models expressing a deficit in this behavior.

This novel study demonstrated for the first time a direct correlation between sensorimotor gating deficits and drug-induced associative learning in the same subject. Furthermore, we revealed that the two, distinct schizophrenia-like models demonstrate divergent sensitivities to the rewarding and incentive motivational properties of Meth. Finally, we determined that augmenting the mGluR5 system altered Meth-induced associative learning in a rodent model of schizophrenia.

Methods

Animals

Male Sprague-Dawley rats were used for all experiments. Rats from the acute (Experiment 1) and repeated, escalating amphetamine studies (Experiment 2) were habituated to the *vivarium* at Rush University Chicago for one week prior to experimentation and were handled at least three times prior to the onset of behavioral experimentation. Rats in the isolation rearing experiment (Experiments 3 and 4) arrived at post natal day 21, which was one day after weaning. Isolation-reared rats were housed alone and social-reared rats were housed in groups of 4 per cage until rats reached approximately 225g, then social

reared rats were housed in groups of 2 per cage prior to behavioral testing. The *vivarium* was maintained at a constant temperature between 23-25°C. All rats were given access to food and water *ad libitum* and were tested during the light cycle (7:00AM to 7:00PM). Experimentation took place during the light cycle of the rats. During this time, rats have been shown to produce the most robust CPP performance to amphetamine (Webb et al. 2009). All studies were conducted with approval by the Rush University Institutional Animal Care and Use Committee in accordance with NIH Guide for the care and use of laboratory animals.

Drugs

(+)Methamphetamine hydrochloride (Meth, NIDA, Bethesda, MD and Sigma Aldrich, St. Louis, MO) and amphetamine sulfate (Amph, Sigma Aldrich) were dissolved in sterile 0.9% saline. Meth was given at a dose of 1mg/kg (as the base). Amph dosage varied between 1 to 8mg/kg (as the base), based on treatment protocol of Peleg-Raibstein and colleagues described below (Peleg-Raibstein et al. 2008). Meth, Amph, and saline vehicle were given at a volume of 1ml/kg and injected intraperitoneally (i.p.). The mGluR5 positive allosteric modulator, 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB; Tocris Bioscience, Ellisville, MO) was given in a suspension of 20% w/v 2-hydroxypropyl-β-cyclodextrin (Sigma Aldrich) in sterile water at a dose of 3mg/kg. The dose of CDPPB used (3mg/kg) most efficiently facilitated extinction learning subsequent to cocaine CPP without affecting motor activity (Gass and

Olive 2009). CDPPB and its vehicle (20% 2-hydroxypropyl-β-cyclodextrin) were given at a volume of 1ml/kg subcutaneously (s.c.).

Behavioral Equipment

Conditioned Place Preference

Small animal activity boxes (Accuscan Instruments, Inc., Columbus, OH) were used for the CPP and motor sensitization evaluations. The boxes (63cm x 30 cm x 30 cm) consisted of two large chambers (25cm x 30cm x 30cm) and one smaller center chamber (13cm x 30cm x 30cm). The two large chambers had unique yet neutral contextual cues of horizontal or vertical white wall stripes (visual cue) and patterned floor with overturned paint dish and grid floor with flat Plexiglas dish (tactile cues). The center chamber had a smooth white floor and white opaque walls. Removable sliding doors separated the compartments. The CPP boxes were equipped with photosensors (24 which measure horizontal movements and 12 which measure vertical movements) and particular patterns in the beam breaks were used to assess motor activity and time spent in each chamber.

Acoustic Startle

Acoustic startle boxes (SR-Lab, San Diego Instruments, San Diego, CA) were equipped with Plexiglas cylinder animal enclosures attached to piezoelectric sensors. A speaker for delivery of acoustic pulses was located above the enclosure. A digital sound level meter (RadioShack, Fort Worth, TX) was used to

assess sound levels using the dB(A) weighted scale and all sound measurements for these studies used this scale.

Experimental Procedures

Thirty min prior to the start of behavioral testing, all rats were habituated to testing room, located in the same suite as the animal *vivarium*. Background white noise of 65dB was present throughout CPP and acoustic startle testing (white noise generator, San Diego Instruments).

Experiment 1.

Timeline for behavioral experiments illustrated in Fig. 21A. Rats were acclimated to startle testing with a baseline startle session. This session included a total of 38 trials presented in a pseudo-random order, including 16 'Pulse Alone' (120dB), six of each 68, 71, and 77dB 'Prepulse + Pulse' trials, and four 'No Stimulus' trails. Four Pulse Alone trials were given at the beginning and end of the test session to allow for a comparison of stable responding and eliminate initial habituation of startle response in each test session (Geyer et al. 1990). These data were not included in the data analysis. Outcomes from the baseline acoustic startle data were used to assign treatment groups. On protocol day 5, half of the rats (n=12) received saline (1ml/kg) and the other half received Amph (3mg/kg) (n=12) 30min prior to the first PPI Test. Each test session consisted of 88 trials, presented in a pseudo-random order, including 24, 120dB Pulse Alone trials, 16 of each 68, 71 and 77dB Prepulse + Pulse trials, and 16 No Stimulus

trials. Again, the four 120dB Pulse Alone trials at the start and end of the test session were excluded from data analysis. A second PPI test was conducted four days later (protocol day 9), wherein the pretreatment was reversed, rats that previously received saline, received Amph and rats that previously received Amph, received saline. Subsequently, rats were tested for Meth-induced CPP. To do so, drug-free rats were first allowed to explore the entire activity box (pre-tested) and time spent in each chamber was determined. As a group, rats had no preference for one chamber over the other (*t*-test, $p>0.05$). However, individual rats demonstrated preference and thus rats were paired with Meth in the chamber in which they spent the least amount of time on the pre-test. Half of the rats were then conditioned with Meth (1mg/kg) on days 14, 16, and 18 by placing rats in the chamber where the least amount of time was spent during pre-test their initially non-preferred side immediately after the Meth injection. On days 15, 17, and 19 rats were placed into the opposite chamber immediately following saline (1ml/kg) administration. The other half of rats were paired with saline on days 14, 16, and 18 and Meth on days 15, 17, and 18. Conditioning lasted for 45min and motor activity was continuously monitored. Three days after the last conditioning session, rats were tested for context preference. The CPP test was conducted by placing untreated rats into the center chamber with the sliding doors shut. The doors were then immediately removed to allow access to the entire CPP box for 30min. Time spent in each chamber and motor activity was monitored. See Fig. 21A for timeline of behavioral procedures.

Experiment 2.

Timeline for behavioral experiments illustrated in Fig. 22A. The 16 rats used for this experiment went through the same acclimation, baseline, and saline pre-treatment startle sessions as in Experiment 1. Four days later, rats were subjected to six days of Amph treatment using the escalating dosing protocol described by Peleg–Raibstein and colleagues (Peleg-Raibstein et al. 2008). On day 1, rats were injected with 1mg/kg Amph at 7AM, 2mg/kg at 1PM and 3mg/kg at 7PM. On day 2, rats received 4mg/kg of Amph at 7AM, 5mg/kg at 1PM and 6mg/kg at 7PM. On day 3, rats were administered 7mg/kg of Amph at 7AM, 8mg/kg at 1PM and 8mg/kg at 7PM. On days 4-6, rats received 8mg/kg of Amph at 7AM, 1PM, and 7PM. PPI test sessions took place 30min after the fifth (second to last) Amph injection (8mg/kg) on the sixth day of injections in order to test rats during the light cycle and to keep time of day for startle sessions consistent. To determine the enduring effects of the escalating Amph treatment on deficits, an additional PPI test was conducted six days after the last Amph treatment. The same test session design was used as in the acute study for each PPI test session. Following PPI testing, Meth-induced CPP was conducted to determine the effects of escalating Amph on reward-mediated learning. Rats were conditioned with Meth and tested for preference (see Fig. 22A, protocol days 25-36) as described in the acute study, however all rats were paired with Meth on the first conditioning day and saline on the second day of conditioning.

Experiment 3.

The protocol timelines for this study are illustrated in Fig. 23A and 24A. After 8 weeks of isolation (n=12) or social (n=12) rearing, rats were subjected to an acoustic startle session (as described for Experiments 1 and 2) after a 30min pretreatment of saline (1ml/kg). This determined the sensorimotor gating deficits associated with rearing conditions. The startle session was repeated on protocol days 56 and 59. This retesting for startle responding was based on reports that isolation reared rats may need to be tested repeatedly in order for a rearing-induced deficit (as indicated by %PPI) to be present (Bakshi et al. 1998). Following these initial startle sessions, three startle sessions (protocol days 70, 78 and 82) were completed after pre-treatment of 0.5, 1.0, or 1.0mg/kg Amph. The conditioned place preference procedure involves components of reward as well as associative learning. Subsequent to Amph startle sessions, rats were subjected to Meth-CPP (protocol days 83-98) as described above for Experiments 1 and 2. One day after the CPP test (protocol day 99), rats were tested again for acoustic startle response after a 30min pretreatment of saline (1ml/kg) to determine the effects that Meth-conditioning had on gating deficits. After 12 days of withdrawal from Meth, rats received Meth (1mg/kg) and then placed in their drug-paired chamber to test for the expression of Meth-induced motor sensitization (see Fig. 24A, protocol day 106). Finally, after 41 days from the original CPP test, rats were tested again for preference immediately after administration of Meth (1mg/kg) (protocol day 139 in Fig. 24A) to test for state-dependent expression of associative learning.

Experiment 4.

Protocol timeline for this study is illustrated in Fig. 25A. After 8 weeks of isolation, 24 rats were tested for sensorimotor gating deficits in two separate startle sessions (protocol days 56 and 59) as in the isolation rearing study. Rats were then subjected to Meth-induced CPP protocols on day 60-71, wherein they received a pre-treatment of the mGluR5 PAM CDPPB (3mg/kg; n=12) or its vehicle (20% 2-hydroxypropyl-β-cyclodextrin, 1ml/kg; n=12) 20min prior to each conditioning session (i.e., before both the Meth and saline pairings). To determine the effects of CDPPB treatment on subsequent sensorimotor gating and motor activity, 24 hr after the CPP Test, rats were subjected to another acoustic startle session (PPI Test 3; protocol day 72). On day 79, rats were given an acute challenge of Meth (1mg/kg) and placed in the chamber where Meth was administered during conditioning (Motor Test). To determine the effects of acutely enhancing mGluR5 signaling on expression of Meth context preference, a second CPP test was conducted on day 85 wherein 20min prior to testing, rats that received CDPPB during conditioning were administered vehicle, and rats that received vehicle during conditioning received CDPPB.

Statistics

%PPI was calculated by the following equation: %PPI = 100 - (Average Startle Magnitude on Prepulse + Pulse Trials / Average Startle Magnitude on Pulse Alone Trials) x 100. Data were analyzed with a two-way repeated

measures analysis of variance (rmANOVA) with *post hoc* Newman Keuls test, α=0.05. An Amph effect score was calculated by averaging the %PPI scores across prepulse intensities per rat and the group average with Amph was subtracted from that of saline pretreatment. CPP was evaluated by comparing time spent in the Meth-paired chamber compared to time spent in the same chamber during the pretest. Preference was defined as a significant increase in time spent in the Meth-paired chamber from during the pre-test to the CPP test using a paired *t*-test, α=0.05. The development of motor sensitization was determined by a paired *t*-test between motor activity during the first and last Meth injection, α=0.05. Motor parameters of horizontal activity, total distance traveled, vertical activity, vertical time, stereotypy count and stereotypy time were chosen to analyze as they reliably represent the motor profile induced by low doses of Meth. Pearson correlation was used to compare %PPI Amph effect score to time spent on the Meth-paired chamber on the CPP test or motor activity ratio (activity on last day of Meth injection / activity on first day of Meth injection). Data are presented as the mean $\pm$ the standard error of the mean (SEM).

Results

Experiment 1.

As shown in Fig. 21B, 3mg/kg i.p. Amph produced deficits in PPI. There was no difference in %PPI response based on the order of Amph administration at any pre-pulse intensity (69, 71 or 77dB) (unpaired *t*-test between rats given

saline on PPI Test 1 and PPI Test 2, $p<0.05$; unpaired *t*-test between rats given Amph on PPI Test 1 and PPI Test 2, $p<0.05$); thus, PPI data under each pre-treatment condition were pooled.

Deficits in %PPI were observed at all pre-pulse intensities tested (68, 71 and 77dB, Fig. 21B) when rats (n=24) were given a 30min pretreatment with Amph (3.0mg/kg) compared to saline (1ml/kg). A two-way rmANOVA resulted in a significant Treatment effect $F_{(1, 46)}=30.504$, $p<0.001$, Pre-pulse Intensity effect $F_{(2, 92)}=34.771$, $p<0.001$ and Treatment x Pre-pulse Intensity interaction $F_{(2, 92)}=10.847$, $p<0.001$ (*post-hoc* Newman Keuls test significance shown in Fig. 21B). Thus, as pre-pulse intensity increased, the %PPI deficit decreased.

Subsequent Meth-conditioning (conducted on protocol days 11-22) revealed that rats (n=22) demonstrated a significant preference for the Meth-paired compartment on the CPP test compared to the pre-test ($t_{(21)}=4.481$, $p<0.001$, Fig. 21C). Since Meth pairing on the first or second day of conditioning did not influence the magnitude of time spent in the Meth-paired chamber during the CPP test (unpaired *t*-test, $t_{(22)}=0.036$, $p=0.972$) Fig. 21C results are the pooled data from both pairing paradigms. To assess whether the magnitude of PPI deficits correlated with the magnitude of CPP, the %PPI Amph effect score (determined from protocol day 5 and 9 data) and time spent on the Meth paired side on the CPP Test (protocol day 22) were compared in the same rat. There was not a correlation between %PPI Amph effect score and CPP Test time spent in the Meth-paired chamber (Pearson correlation, $r=0.081$, $p=0.721$, Fig. 21D). These

data show that responding to a single dose of Amph did not induce sensorimotor gating deficits that predicted responding to Meth-induced CPP.

The conditioning procedure also resulted in the development of motor sensitization to Meth in horizontal activity ($t_{(21)}$=3.227, p=0.004) and stereotypy count ($t_{(22)}$=2.376, p=0.027) parameters (Table. 8). To determine the correlation between sensorimotor gating deficits and subsequent sensitization to Meth, the horizontal motor activity ratio (activity on the last / first Meth injection) was compared to the %PPI Amph effect score. There was a significant, positive correlation between horizontal motor activity ratio and %PPI Amph effect score (Pearson correlation, r=0.433, p=0.044, Fig.21E) indicating that rats with larger deficits in sensorimotor gating exhibited more robust sensitized motor response to Meth. Correlation assessments were also performed on stereotypy count data, as this parameter demonstrated sensitization in this cohort of rats. However, there was not a significant correlation for the stereotypy count data set (data not shown).

Experiment 2.

Testing for Amph (8mg/kg i.p.)-induced PPI deficits on the last day of the repeated, escalating Amph administration protocol (PPI Test 2 on protocol day 14; Fig. 22A) revealed deficits in %PPI as compared to saline (PPI Test 1) at 71 and 77dB pre-pulse intensities. Two-way rmANOVA revealed a significant Treatment effect $F_{(1, 30)}$=4.396, p=0.045, a significant Pre-pulse Intensity effect $F_{(2,60)}$=60.801, p<0.0001 and no Treatment x Pre-pulse Intensity interaction

$F_{(2,60)}$=2.519, p<0.089 (*post hoc* Newman Keuls test, p<0.01, Fig. 22B). Subsequent Meth conditioning revealed that rats had a significant preference for the Meth-paired chamber (paired *t*-test, $t_{(15)}$=3.936, p<0.01, Fig. 22C). Based on the PPI results generated on protocol day 5 and 14 and CPP results from protocol day 36, there was a significant correlation between %PPI Amph effect score and CPP Test time spent in the Meth-paired chamber (Pearson correlation, r=-0.588, p=0.017, Fig. 22D). That is, the rats that had low sensorimotor gating deficits had a greater preference for the context associated with the rewarding effects of Meth. Additionally, these data indicate that repeated administration of Amph induces PPI deficits that inversely correlate with the strength of Meth-induced CPP.

Development of motor sensitization, determined by comparing the first and last Meth injection, did not occur during conditioning to any parameter measured, due to the repeated Amph treatment history (paired *t*-test, p>0.05; Table 9). There was also no correlation between the horizontal motor activity ratio (activity on day of last / activity on day of first Meth injection) and %PPI Amph effect score (Pearson correlation, r=-0.027, p=0.919, Fig. 22E).

Experiment 3.

After eight weeks of isolation or social rearing, rats were tested for deficits in %PPI with saline pretreatment (PPI Tests 1-3, Fig. 23A). No differences were observed between isolation and social reared groups (two-way rmANOVA, p>0.05, data not shown). Amph administered 30min prior to the startle test

session did not induce sensorimotor gating deficits that distinguish isolation (PPI Tests 3-6, two-way rmANOVA with *post-hoc* Newman Keuls test, $p>0.05$, data not shown). Rats were subsequently conditioned with Meth (see Fig. 23A). Motor sensitization was assessed during conditioning and place preference was subsequently assessed three days later in a drug-free state. On conditioning day 1, isolation compared to social reared rats demonstrated an increase in horizontal activity (paired *t*-test, $t_{(20)}=2.268$, $p=0.035$) and total distance (unpaired *t*-test, $t_{(20)}=3.962$, $p=0.001$) (Table 10). Therefore, rearing history plays a role in the acute motoric response to Meth. Furthermore, it can be seen that the motor responses subsequent to the initial Meth injection in the repeated, escalating Amph treated rats (Experiment 2, Table 9) are heightened compared to social reared rats (Table 10). Though different treatment conditions were employed to induce a schizophrenia-like brain state, common outcomes, such as enhanced response to an acute treatment of Meth, can occur. Both social and isolation reared rats developed motor sensitization to Meth over the course of conditioning sessions (Table 10). There is not a difference between social and isolation reared rats in the magnitude of motor sensitization development.

Social but not isolation-reared rats demonstrated a significant preference for the Meth-paired chamber after conditioning compared to the amount of time spent in the same chamber during the pre-test (unpaired *t*-test, $t_{(11)=}2.756$, $p<0.05$, Fig. 23B&C). The data were similar to those obtained from the Amph models of schizophrenia (compare with Fig. 21C and 22C).

To determine if rearing history in conjunction with Meth conditioning differentially influenced PPI, rats were tested for acoustic startle response after the CPP test (i.e., on day 99; see Fig. 23A). For this PPI test, all rats were subjected to a 30min pretreatment with saline. Isolation reared rats had a lower %PPI score at the 71 and 77dB pre-pulse intensity. A two-way rmANOVA resulted in a Rearing Condition effect that was close to significance $F_{(1,19)}=4.313$, $p=0.052$, a significant Pre-pulse Intensity effect $F_{(2,38)}=51.172$, $p<0.0001$, and no effect of Rearing Conditioning x Pre-pulse Intensity interaction $F_{(2,38)}=0.215$, $p=0.807$. Because the Rearing effect demonstrated a trend and there was a significant effect of Pre-pulse Intensity, a *post hoc* test was conducted (*post-hoc* Newman Keuls test, $p<0.05$, Fig. 23D).

On day 106 (which was 12 days after the last Meth conditioning session; refer to Fig. 24A), all rats were habituated to the Meth-paired chamber, then given a Meth injection (1mg/kg), and immediately placed back into the Meth-paired chamber. Because of the significant effect of rearing condition to the acute response of Meth demonstrated by the total distance parameter (Table 10), a time course evaluation of motor expression was conducted. For total distance, a two-way rmANOVA resulted in a no Rearing Condition effect $F_{(1,19)}=3.547$, $p=0.075$, and significant Time effect $F_{(8,152)}=7.950$, $p<0.0001$ and Rearing Condition x Time interaction $F_{(8,152)}=2.345$, $p=0.021$. A *post-hoc* Newman Keuls test revealed condition differences at 5, 30, and 35min after Meth injection ($p<0.05$, Fig. 24B). As rats are injected (i.p.) and immediately placed in the activity box, the hyperactivity that resulted at 5min in the isolation reared rats is likely due to a

heightened conditioned motor response and/or rearing condition alone. Rats reared in isolation for at least four weeks demonstrate increased motor activity, in general, compared to social reared counterparts (Bakshi and Geyer 1999). Also, these data demonstrate that during the time of peak Meth effects (30-35min), the rearing condition may have also influenced the expression of Meth-induced motor sensitization.

In contrast to the rearing condition differences in motor responding to an acute challenge of Meth, place preference measure during an acute Meth challenge did not distinguish the two groups. That is, on day 139 (Fig. 24A), when rats were tested for preference immediately after an administration of Meth (1mg/kg), both the social-reared (unpaired *t*-test, $t_{(20)}=4.72, p<0.001$, Fig. 24D) and the isolation-reared (unpaired *t*-test, $t_{(20)}=5.01$, $p<0.001$, Fig. 24E) rats demonstrated a preference for the Meth paired side. These data demonstrate that isolation reared rats were able to express Meth-induced associative learning after they were primed with the drug cue.

Experiment 4.

Comparing findings from Experiment 3 and 4 (i.e., data from Fig. 23C and Fig. 24D) indicated that isolation-reared rats can acquire CPP tasks and this is expressed if a drug cue (i.e., the unconditioned stimulus) was present during testing for the salience of the paired context (i.e., the conditioned stimulus). This suggested that if the salient signal strength is enhanced during conditioning, isolation-reared rats should be able to express preference in a drug-free state. As

augmenting mGluR5 activity with a PAM facilitates extinction learning of cocaine-induced CPP (Gass and Olive 2009), we hypothesized that a mGluR5 PAM should be able to boost association learning during Meth conditioning, and in so doing, aid isolation-reared rats to acquire the task. Therefore, as shown in Fig. 25A, isolation reared rats were administered the mGluR5 PAM, CDPPB (3mg/kg, n=11) or its vehicle (1ml/kg, n=11) prior to conditioning and then subsequently tested for preference (CPP Test 1, Fig. 25A). Here, as we hypothesized, isolation-reared rats demonstrated preference for the Meth-paired context when tested in the drug free state (Fig. 25B, gray bars). To determine if acute mGluR5 activation altered the expression of Meth-induced CPP, these rats were retested (see Fig. 25A, CPP Test 2; protocol day 85) 20min after CDPPB (3mg/kg, n=11) or vehicle (1ml/kg, n=11) administration. CDPPB enhanced preference for the Meth-paired chamber in rats that received vehicle pre-treatment during conditioning (Fig. 25B, left set of gray and black bars) and this enhancement reached the level obtained in rats that received CDPPB during conditioning.

Rats were tested for sensorimotor gating deficits one day following CPP Test 1 in a drug free state (protocol day 72, see Fig. 25A). It was determined that there was no difference in %PPI between isolation reared rats given a treatment of CDPPB or its vehicle prior to conditioning sessions (protocol days 63-68) on PPI Test 3 in a drug-free state (two-way rmANOVA, non significant effect of Treatment: $F_{(1,17)}=0.005$, $p=0.942$, a significant effect of Pre-Pulse $F_{(2,34)}=45.052$, $p<0.0001$, and a non significant Interaction $F_{(2,34)}=1.154$, $p=0.327$; Fig. 25C).

Therefore, while CDPPB enhanced development and expression of Meth-induced CPP, it had no effect on sensorimotor gating deficits. It was also determined that there was no difference between CDPPB or vehicle treatments during conditioning on motor activity to a challenge administration of Meth (1mg/kg, i.p.) after extended withdrawal on protocol day 79 (two-way rmANOVA with *post hoc* Newman Keuls, $p>0.05$, data not shown). Therefore, while CDPPB enhanced development and expression of Meth-induced CPP, it had no effect on sensorimotor gating deficits or on motor activity induced by Meth in isolation reared rats.

Discussion

The current study provides several novel findings, including the following: 1) Both pharmacological and developmental rodent models of schizophrenia demonstrated deficits in Meth-induced associative learning. 2) Isolation-reared rats were able to express Meth CPP in a state-dependent manner. 3) The mGluR5 system was critical for the acquisition and expression of Meth-induced associative learning in isolation-reared rats.

Enhanced motor activity occurs in schizophrenia-like rodent models.

Results from Experiments 1 through 3 demonstrated that schizophrenia-like rodents were more sensitive to the motoric properties of Meth. In Experiment 1, sensorimotor gating deficits induced by an acute administration of Amph correlated positively with Meth-induced motor sensitization.

Demonstrated in Experiment 3, isolation reared rats showed greater motor activation to an acute administration of Meth compared to their social reared counterparts. Rats administered with repeated, escalating Amph (Experiment 2) were also hyperactive in response to the first Meth injection compared to social reared control animals. Furthermore, the level of sensitized motor responding to an acute Meth challenge following withdrawal from repeated exposure to Meth was greater in Isolation reared rats compared to social reared rats (Experiment 2). Hyperactivity in response to Amph and Meth administration is a well-documented feature of post-weaning isolation in rodents (Dai et al. 2004;Smith et al. 1997). Because the isolation-reared rats used in the current study did not display initial deficits in %PPI, (note: PPI deficits do not occur 100% of the time in these animals (Fone and Porkess 2008)) it was important to demonstrate this sensitivity to acute administration of Meth,. These findings are in alignment with the neonatal ventral hippocampal lesion (NVHL), schizophrenia model in which enhanced cocaine- and nicotine-induced motor sensitization occurs (Berg and Chambers 2008;Chambers and Taylor 2004). An increased sensitivity to the motoric properties of Meth also parallels the human condition in which amphetamines exacerbate psychosis in schizophrenia patients (Angrist et al. 1980;Janowsky and Davis 1976).

Sensorimotor gating deficits predict magnitude of Meth-induced associative learning.

Results from the repeated, escalating Amph treatment (Experiment 2) and isolation rearing experiments (Experiment 3) converge to demonstrate that schizophrenia-like rats show deficits in Meth-induced associative learning. We revealed that sensorimotor gating deficits induced pharmacologically (Peleg-Raibstein et al. 2008) inversely correlated with Meth-conditioned contextual preference. These results suggest that schizophrenia-like rats are less sensitive to the rewarding properties of Meth.

Because of the potential confound of cross-sensitization between Amph and Meth (Hall et al. 2008), we chose to validate the repeated, escalating Amph findings using the developmental rodent model of schizophrenia, isolation rearing. We determined that isolation-reared rats failed to express Meth-induced associative learning (Experiment 3). This finding is in keeping with our results for the repeated, escalating Amph experiment and the literature in which isolation reared rats and NVHL rats fail to express Amph-induced CPP (Le et al. 2002;Wongwitdecha and Marsden 1995). There are two likely explanations for these findings due to the nature of the CPP assessment: 1) schizophrenia-like rats are deficient in the ability to perceive reward, or 2) schizophrenia-like rats have deficits in mnemonic processes that result in a failure to express learned associations between contextual cues conditioned with Meth.

Another novel finding of the current study is that %PPI was decreased in isolation-reared rats compared to social-reared rats after Meth conditioning.

Prior work demonstrated that isolation-reared rats have increased sensorimotor gating deficits in response to a sensitizing regimen of Meth (Dai et al. 2004). We extended this finding to reveal that these deficits occur in drug-free, Meth-conditioned rats thus supporting our hypothesis that Meth-induced CPP is negatively associated with sensorimotor gating deficits.

Reward and associative learning are both measured in Meth-induced CPP. These properties are also associated with negative symptoms of schizophrenia (i.e., anhedonia, cognitive fragmentation, and working memory deficits). NVHL rodents will expend more effort than unlesioned controls in order to receive the Meth reinforcer (Brady et al. 2008). This suggests that schizophrenia-like rodents are more motivated by the reinforcing properties of Meth. Therefore, it may be hypothesized that schizophrenia-like rodents are capable of experiencing Meth reward but may be less sensitive to these properties. There are conflicting reports on spatial learning and memory abilities in rodent models of schizophrenia including isolation rearing. Isolation-reared rats show an enhanced ability to locate a submerged platform in the Morris water maze experiment demonstrating superior acquisition of spatial learning over social-reared counterparts (Wongwitdecha and Marsden 1995). However, others report no differences in acquisition in Morris water maze training (Quan et al. 2010;Schrijver et al. 2002), or that isolation-reared rats take longer to find the platform indicating deficits in multiple phases of the learning process (Dai et al. 2004;Hellemans et al. 2004). Results for spatial memory retention are also varied, with reports demonstrating no difference between rearing conditions

(Hellemans et al. 2004;Schrijver et al. 2002) or a deficit in isolation reared rats (Quan et al. 2010). There results suggest that isolation rearing rodents may exhibit working memory deficits in the acquisition or expression phase of mnemonic processes. Therefore, spatial working memory necessary to associate an environmental context with the rewarding properties of a stimulant may be compromised in schizophrenia-like rats. Cognitive assessments in non-human primates given repeated, escalating doses of Amph show deficits in some aspects of working memory but not in acquisition of visual discrimination tasks that assess associative learning between contextual cues and food reward (Castner et al. 2005). Working memory deficits and anhedonia in schizophrenia patients are well documented (Driesen et al. 2008;Park and Holzman 1992). Therefore, these negative symptoms may be assessed in the CPP task in schizophrenia-like rodents.

Meth and mGluR5 PAM administration enhances associative learning performance in isolation- reared rats.

We observed that when isolation-reared rats were administered Meth prior to a CPP test, the animals were capable of expressing a preference for the context previously paired with Meth (Experiment 3). These novel findings suggest that isolation-reared rats can demonstrate that associative learning had occurred when they were tested in the presence of the unconditioned stimulus. The addition of the drug cue during the CPP Test replicates the physiological state that occurred during conditioning and increases the ability of the

schizophrenia-like rodent to express the previously acquired association. Therefore, schizophrenia patients experiencing negative symptoms including anhedonia and working memory deficits may be less sensitive to the rewarding properties of stimulants, but when conditions are appropriate, they may be able to make stimulant-induced associations.

The mGlu5 receptor is critical for the development of Meth-induced associative learning (Miyatake et al. 2005) and for the facilitation cocaine-induced place preference extinction learning (Gass and Olive 2009). Our findings demonstrate that mGlu5 receptors were also critical for both the acquisition and expression of Meth-induced CPP in a rodent model of schizophrenia. Our results also suggest that the magnitude of preference for the Meth-paired context was more enhanced by pre-conditioning treatment with the mGluR5 positive allosteric modulator, as rats that were administered CDPPB prior to conditioning spent more time on the Meth-paired chamber compared to rats that were administered CDPPB on the CPP Test day. One explanation for our findings is that CDPPB administration compensated for a deficient mGluR5 system in isolation-reared rats. Isolation rearing decreases levels of mGluR5 in the prefrontal cortex (Gregory and Szumlinski 2008;Melendez et al. 2004) a brain region essential for working memory and executive function. The mGluR5 PAM is also known to enhance acquisition of extinction learning of cocaine-induced place preference (Gass and Olive 2009). These evidences converge to indicate that mGlu5 receptors contributed to the positive results obtained with the Meth 'state-dependent' CPP test in the isolation-reared rats pre-treatment in

the current study. This possibility was corroborated by the ability of isolation-reared rats to demonstrate Meth-induced CPP following treatment with the mGluR5 NAM, CDPBB during conditioning. Therefore, when associative learning signals are strengthened in schizophrenia-like rats *via* Meth or an mGluR5 PAM, a preference for cues previously paired with Meth can be subsequently expressed.

In summary, the results of the current study converged to demonstrate that sensorimotor gating deficits are inversely correlated with Meth-induced associative learning, whereas sensitivity to motor activity is enhanced in schizophrenia-like rats. We further demonstrated isolation reared rats could express state-dependent CPP and that mGluR5 is a critical mediator for both acquisition and expression of Meth-induced associative learning. Both working memory and anhedonia predict substance abuse likelihood in schizophrenia patients (Potvin et al. 2008a;Potvin et al. 2008b). Thus, the results of the current study suggest that sensorimotor gating deficits may predict the propensity of schizophrenia patients to for Meth-induced associative memories.

Tables

Table 8. Motor responses to Meth after acute Amph treatment.

Motor Parameter	First Injection	Last Injection
Horizontal Activity	4991±254	6106±285**
Total Distance (cm)	1095±54	1314±127
Vertical Activity	1622±178	1468±165
Vertical Time (sec)	689±86	656±87
Stereotypy Count	3006±188	3748±254*
Stereotypy Time (sec)	526±35	631±44

For this study, Amph was given on protocol days 5 or 9; the first and last Meth injections were given on protocol days 14 or 15 and 18 or 19, respectively. Data are number of beam breaks, distance (cm) or time (sec) detected by photobeams in the small animal activity box used for CPP. Data represent mean ± SEM. n=20-23. Paired *t*-test, *p<0.05, **p<0.01.

Table 9. Motor responses to Meth after repeated, escalating Amph treatment.

Motor Parameter	Meth Conditioning	
	First Injection	Last Injection
Horizontal Activity	7416±416	7981±374
Total Distance (cm)	1546±121	1678±130
Vertical Activity	1696±103	1616±156
Vertical Time (sec)	566±48	735±146
Stereotypy Count	4981±369	5369±367
Stereotypy Time (sec)	804±53	846±62

For this experiment, Amph was given in an escalating dosing paradigm (1 to 8 mg/kg, i.p.) on protocol days 9 to 14. The first and last Meth injections were administered on protocol days 28 and 32, respectively. Data are number of beam breaks, distance (cm) or time (sec) detected by photobeams in CPP box. Data represent mean ± SEM. n=13-16. Paired *t*-test, $p>0.05$.

Table 10. Motor responses to Meth following social or isolation rearing.

Motor Parameter	Social Rearing		Isolation Rearing	
	First Injection	Last Injection	First Injection	Last Injection
Horizontal Activity	4365±250	5460±339*	5305±308†	6136±411
Total Distance (cm)	924±53	1223±123*	1416±106††	1780±162
Vertical Activity	1459±116	1709±199	1840±262	1363±144*
Vertical Time (sec)	559±54	670±87*	748±116	574±64
Stereotypy Count	2487±226	3399±311**	2900±179	3893±279**
Stereotypy Time (sec)	417±40	567±55**	477±27	701±56**

For this experiment, the first and last Meth injections were administered on protocol days 90 and 94, respectively. Data are number of beam breaks, distance (cm) or time (sec) detected by photobeams in CPP box. Data represent mean ± SEM. Social n=11-12, Isolation n=11. Paired *t*-test within Social or Isolation Rearing groups, *p<0.05, **p<0.01. Unpaired *t*-test between Social and Isolation Rearing groups, †p<0.05, ††p<0.01.

Table 11. Motor responses to Meth by rats reared in isolation following pre-treatment of CDPPB or its vehicle.

Motor Parameter	Vehicle		CDPPB	
	First Injection	Last Injection	First Injection	Last Injection
Horizontal Activity	4282±282	5285±315**	4256±289	5619±293***
Total Distance (cm)	1131±105	1401±167*	1097±106	1301±134*
Vertical Activity	1639±134	1760±197	1528±124	1959±181*
Vertical Time (sec)	642±76	695±94	642±75	1080±246
Stereotypy Count	2283±568	2976±249**	2193±174	3145±208***
Stereotypy Time (sec)	391±28	492±39**	381±31	513±33**

For this experiment, the first and last Meth injections were administered on protocol days 63 (Day 1) and 67 (Day 5), respectively. Data are number of beam breaks, distance (cm) or time (sec) detected by photobeams in CPP box. Data represent mean ± SEM. Vehicle (1ml/kg, s.c.) n=11-12, CDPPB (mGluR5 PAM, 3mg/kg, s.c.) n=11-12. Paired *t*-test within Social or Isolation Rearing groups, *p<0.05, **p<0.01, ***p<0.001.

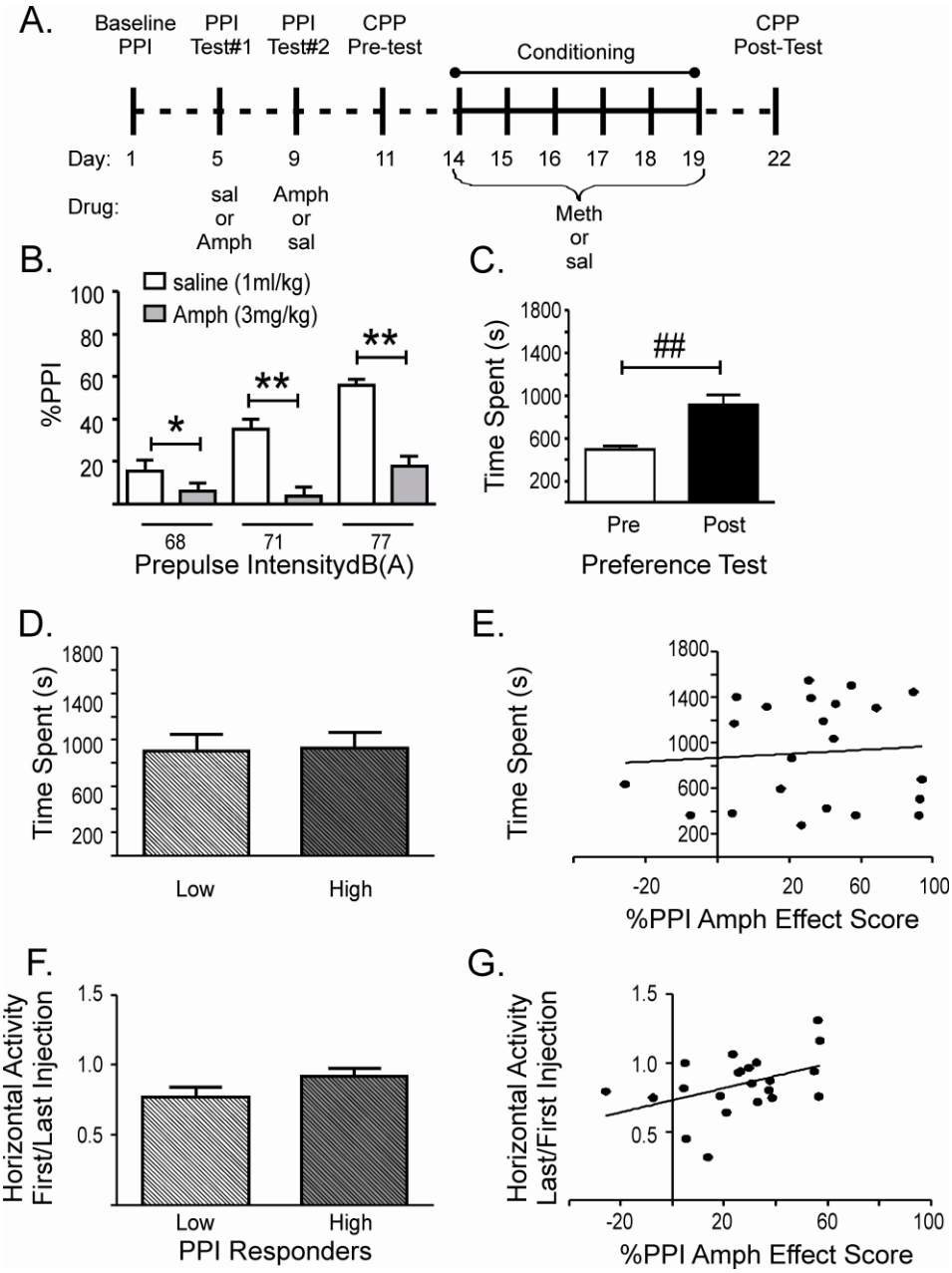

Figure 21. Deficits in sensorimotor gating induced by acute administration of Amph predict motor but not reward responses to Meth.

A) Timeline of behavior/treatment protocol for Amph-induced deficits in prepulse inhibition (PPI) assessments (protocol days 1-11) and Meth-induced conditioned place preference (CPP; protocol days 14-22). On day 1, all rats (n=22) were tested with a baseline startle session. On days 5, half of the rats were given a 30min pretreatment with saline (1ml/kg, i.p.) and the other half were given Amph (3mg/kg, i.p.) and then subjected to the startle session. On day 9 the treatment groups were switched and rats that previously received saline received Amph prior to the startle session. Rats were then pre-tested (protocol day 11) and conditioned with Meth every other day for six days (protocol days 14-19). Rats were tested for place preference on protocol day 22. **B)** PPI Test 1 and 2 (protocol days 5 and 9). Data represented here are pooled based on pre-PPI Test treatment history. Rats demonstrated a significant deficit in %PPI 30min after a pre-treatment of Amph compared to saline at 68, 71 and 77dB prepulse intensities (two-way rmANOVA with *post hoc* Newman Keuls test, *$p<0.05$, **$p<0.01$). **C)** CPP Test (protocol day 22). Rats showed a significant increase in time spent in the Meth-paired chamber after conditioning compared to the pre-test (unpaired *t*-test, ##$p<0.01$). **D)** There was no correlation for CPP magnitude (i.e., Time spent in the Meth-paired compartment) vs. the %PPI Amph Effect Score. **E)** There was a significant correlation between the development of motor sensitization and the %PPI Amph Effect Score (Pearson correlation, $r=0.433$, $p=0.044$). Motor sensitization as assessed by a ratio of horizontal activity on the first over the last injection of Meth during the

conditioning phase. Sal = saline, 1ml/kg, i.p.; Amph = amphetamine, 3mg/kg, i.p.; Meth= methamphetamine, 1mg/kg, i.p.

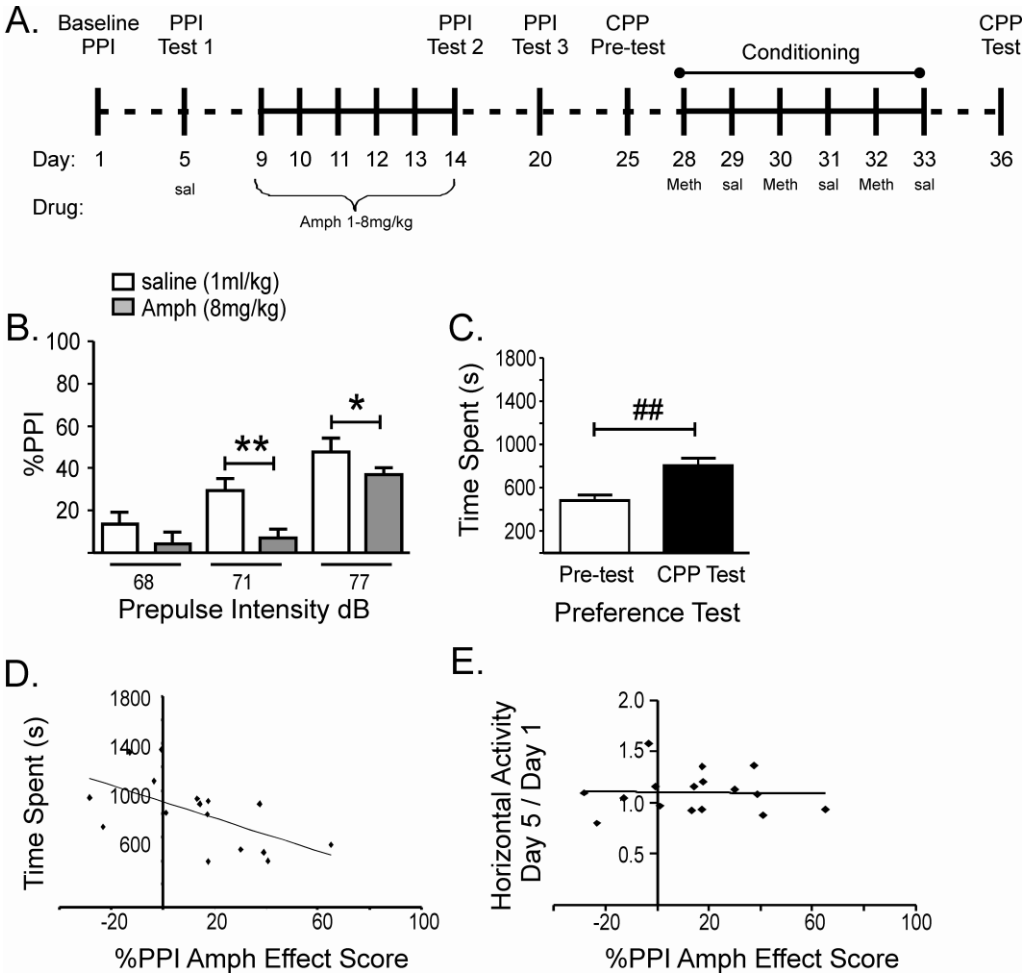

Figure 22. Deficits in sensorimotor gating induced by a repeated, escalating administration of Amph predict reward, but not sensitized motor, responses to Meth.

A) Timeline of behavior/treatment protocol for Amph-induced deficits in prepulse inhibition (PPI) assessments (protocol days 1-20) and Meth-induced conditioned place preference (CPP; protocol days 25-36). On day 1, all rats (n=16) were tested with a baseline startle session. On protocol day 5, rats were subjected to an acoustic startle session. On protocol days 9-14, rats received 3 once-daily injections of Amph that escalated from 1-8mg/kg. On the last day of

Amph treatment, rats were tested for acoustic startle response and on protocol day 20 in a drug-free state. Rats were pre-tested on protocol day 25 then conditioned with Meth or saline on protocol days 28-33. On protocol day 36, rats were tested for place preference. **B)** PPI Test 1 (protocol day 5). Rats demonstrated a significant decrease in %PPI when given a 30min pretreatment of Amph (8mg/kg, i.p., day 14, PPI Test 2) compared to a saline (1ml/kg, i.p.) pretreatment at 71 and 77dB (two-way rmANOVA, with *post hoc* Newman Keuls test, *$p<0.05$, **$p<0.01$). **C)** CPP Test (protocol day 36). Rats demonstrated a significant preference for the Meth-paired chamber after conditioning (unpaired *t*-test, ##$p<0.01$). **D)** There was a significant correlation between amount of time spent on the Meth-paired compartment during the CPP Test and the %PPI Amph effect score (Pearson Correlation, $p<0.05$, $r^2=0.3451$). **E)** There was no correlation between the development of motor sensitization as assessed by a ratio of horizontal activity on the first over the last injection of Meth and the %PPI Amph Effect Score (Pearson correlation, $p>0.05$, $r^2=0.001$). Sal = saline, 1ml/kg, i.p.; Amph = amphetamine, 1-8 mg/kg, i.p.; Meth= methamphetamine, 1mg/kg, i.p.

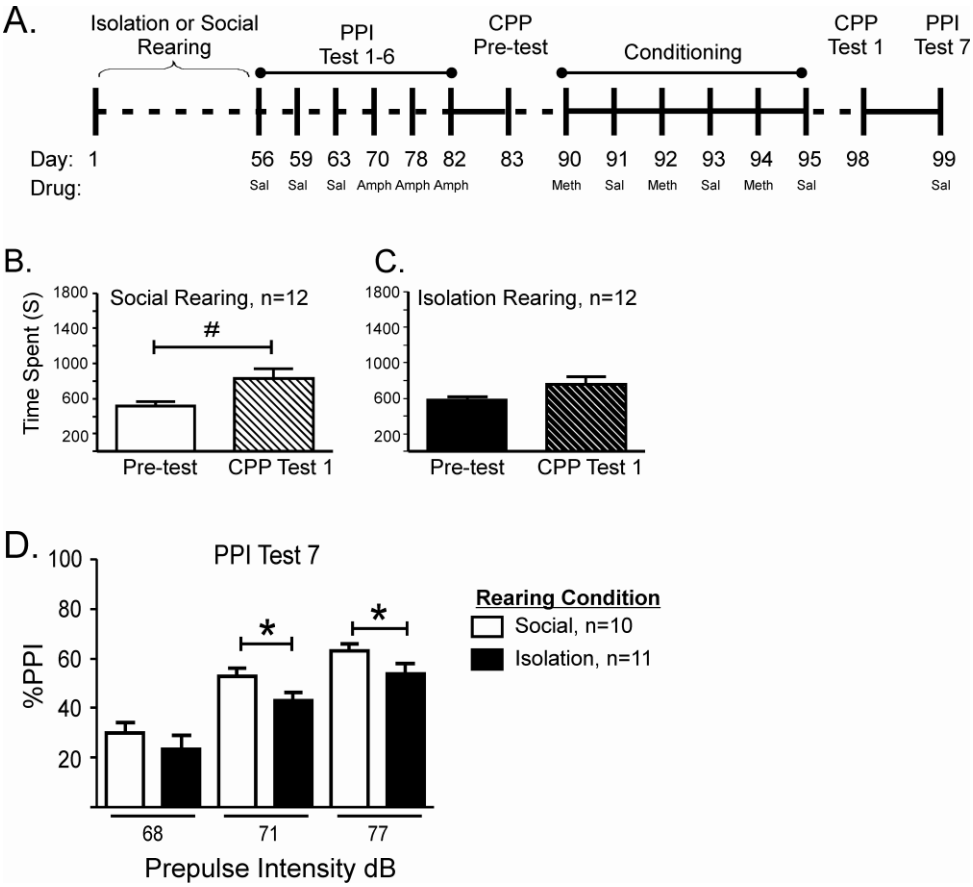

Figure 23. Isolation rearing produced sensorimotor gating and associative learning deficits following Meth conditioning.

A) Timeline of behavior/treatment protocol. After 8 weeks of isolation or social rearing (protocol days 1-56), all rats were tested for acoustic startle response 30min following a saline pretreatment (1ml/kg, i.p.; protocol days 56 and 59). On days 70, 78, and 82, rats were tested for startle 30min after an Amph (0.1, 1 and 1mg/kg, i.p., respectively) pretreatment. Rats were tested for initial preference on protocol day 83, conditioned with Meth (1mg/kg, i.p.) on protocol days 90, 92, and 94, and saline (1ml/kg, i.p.) on protocol days 92, 93, and 95. Rats were subsequently tested for preference on protocol day 98 (CPP Test 1).

Rats were tested for acoustic startle response after a saline (1ml/kg) administration on day 99 (PPI Test 7). **B)** Social reared rats demonstrated a significant preference for the Meth-paired CPP chamber after conditioning compared to the CPP pre-test (unpaired *t*-test, #p<0.05). **C)** Isolation reared rats did not demonstrate a preference for the Meth-paired environment in a drug-free state on CPP Test 1. **D)** After Meth conditioning, isolation reared rats demonstrated a significant deficit in %PPI compared to social reared rats at the 71 and 77dB prepulse intensity (two-way rmANOVA with *post hoc* Newman Keuls test, *p<0.05). Sal = saline, 1ml/kg, i.p.; Amph = amphetamine, 0.1-1mg/kg, i.p.; Meth= methamphetamine, 1mg/kg, i.p.

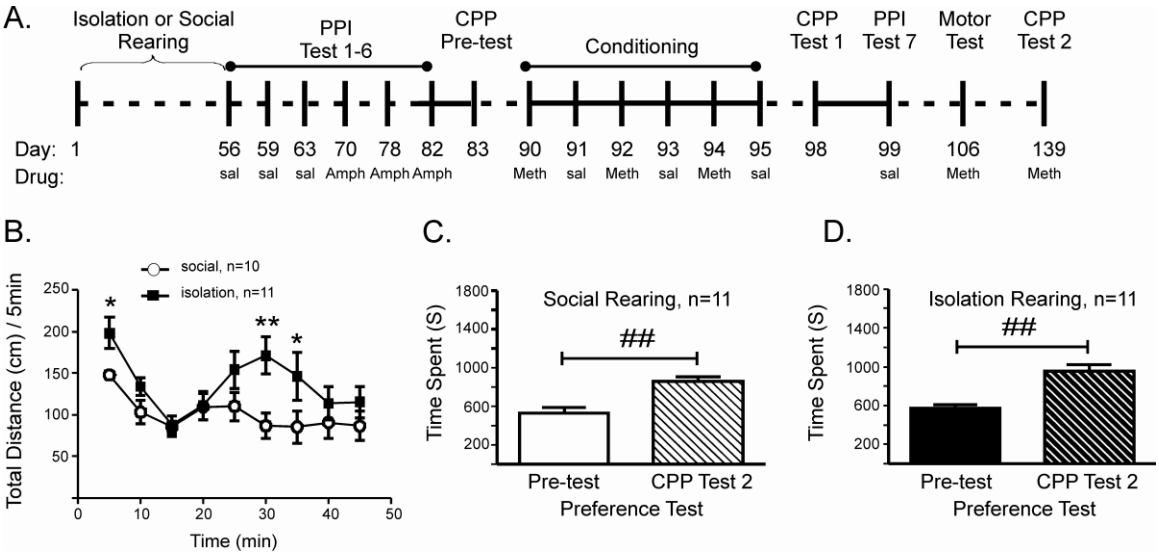

Figure 24. Isolation reared rats demonstrated enhanced hyperactivity and associative learning in response to Meth administration.

A) Timeline of behavior/treatment protocol; days 1-99 are similar to that described in Fig. 3. On protocol day 106, all rats were given a Meth injection (1mg/kg, i.p.) on the Meth-paired chamber and motor activity was monitored (Motor Test). Finally, rats were tested for state-dependent CPP (CPP Test 2; protocol day 139) immediately following an injection of Meth (1mg/kg, i.p.). **B)** Isolation reared rats demonstrated an increase in total distance traveled (cm) at 5, 30 and 35min post-Meth (1.0mg/kg, i.p.) injection (two-way rmANOVA with *post hoc* Newman Keuls test, *p<0.05, **p<0.01) compared to social reared rats. Both social **C)** and isolation **D)** reared rats demonstrated a significant preference for the Meth-paired compartment post- compared to pre-conditioning when given an injection of Meth (1.0mg/kg, i.p.) immediately before CPP Test 2

(unpaired *t*-test, ##p<0.01). Sal = saline 1ml/kg, i.p.; Meth = methamphetamine, 1mg/kg, i.p.

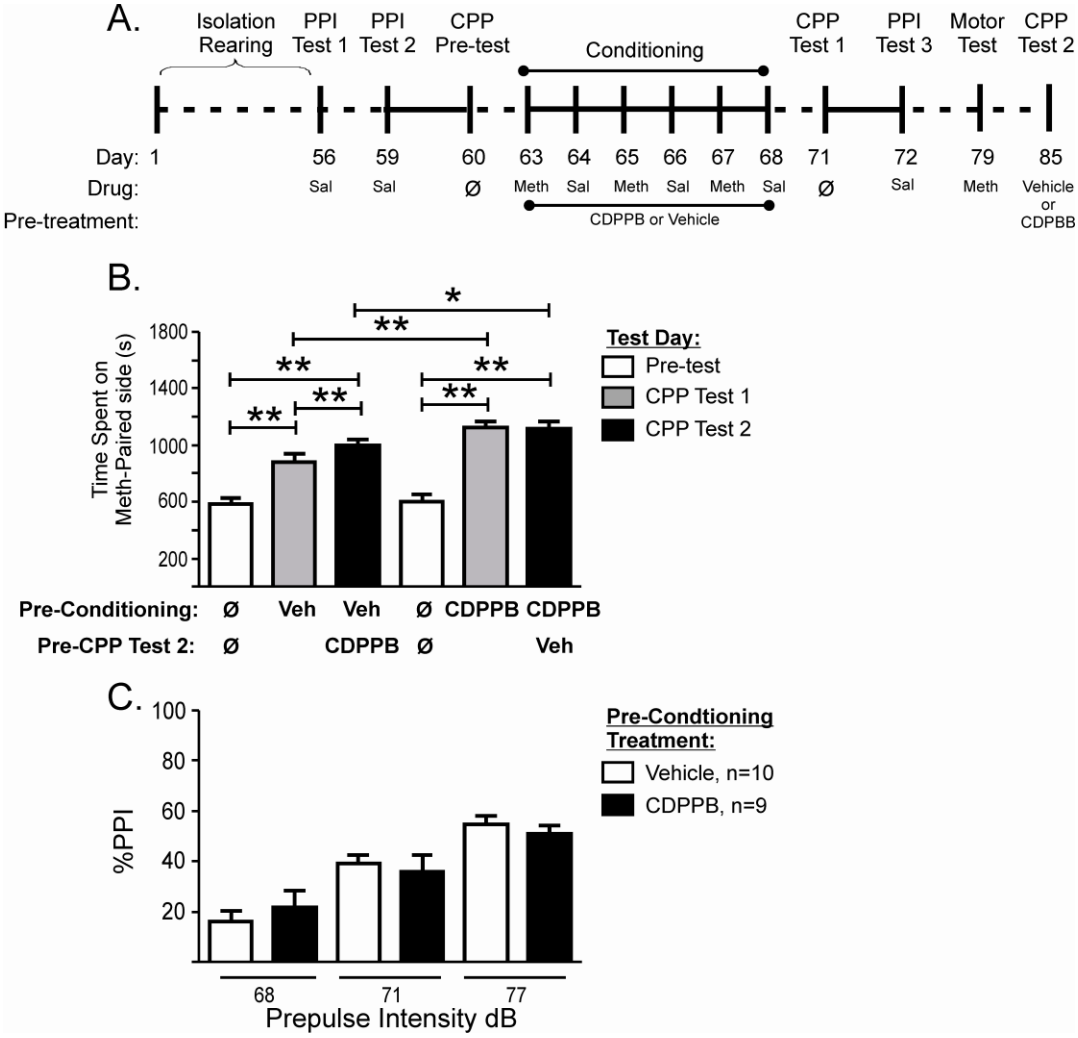

Figure 25. Activation of mGluR5 enhances development and expression of Meth-induced CPP in isolation reared rats without an effect on subsequent sensorimotor gating deficits.

A) Timeline of behavior/treatment protocol. Rats were reared in isolation for 8 weeks and subsequently tested for sensorimotor gating deficits on protocol days 56 and 59 (PPI Tests 1 and 2). Rats were pre-tested on protocol day 60 in a drug-free state. During conditioning, rats were given a 20min pre-treatment with

CDPPB (3mg/kg, s.c.; n=11) or its vehicle (1ml/kg, s.c.; n=11) prior to Meth (1mg/kg, i.p.) on days 63, 65, and 67, or saline (1ml/kg, i.p.) on days 64, 66, and 68. Rats were tested for Meth-induced preference on day 71 in a (CPP Test 1) in a drug-free state and sensorimotor gating deficits on day 72 (PPI Test 3) 30min after a saline (1ml/kg, i.p.) injection. Rats were given an injection of Meth 1mg/kg and immediately placed in the Meth-paired chamber as in conditioning on the Motor Test (day 79). Rats administered CDPPB (3mg/kg, s.c.) prior to conditioning were given vehicle (1ml/kg, s.c.) and rats that had a pre-conditioning treatment of vehicle (1ml/kg, s.c.) were injected with CDPPB (3mg/kg, s.c.) prior to CPP Test 2 on protocol day 85. **B)** CPP (protocol days 71 and 85). Rats administered CDPPB during conditioning demonstrated greater amount of time spent on the Meth-paired chamber compared to rats given pre-conditioning vehicle. Rats administered CDPPB prior to CPP Test 2 spent more time on the Meth-paired side on this test day compared to CPP Test 1. Rats administered CDPPB prior to conditioning demonstrated an increase in time spent in the Meth-paired side on compared to rats that were administered CDPPB prior CPP Test 2 on this test day (two-way rmANOVA with *post hoc* Newman Keuls test, *$p<0.05$, **$p<0.01$). **C)** There was no difference between rats given CDPPB or vehicle prior to conditioning on %PPI in the acoustic startle response session on day 72 (PPI Test 3) (two-way rmANOVA with *post hoc* Newman Keuls test, $p>0.05$).

CHAPTER VIII

GENERAL DISCUSSION

The overall goal of this dissertation project was to expand our knowledge of the role of the mGluR5 system in Meth addiction and in the co-morbidity of schizophrenia and Meth substance use disorder. Towards that end, we characterized the function and expression of mGluR5 following both short (Chapters III and V) and extended (Chapters IV and V) periods of withdrawal after repeated Meth administration. We determined that mGluR5 was necessary for the maintenance of Meth-induced place preference (Chapter IV) and that mGluR5 expression was differentially regulated by Meth in various reward-related brain regions (Chapters V and VI). We found that sensorimotor gating deficits associated with schizophrenia were negatively correlated with Meth-induced associative learning in both pharmacological and developmental rat models of schizophrenia (Chapter VII). Both models also showed hyperactive motor responses to Meth compared with controls (i.e., social rats; Chapter VII). Finally, we determined that activating mGluR5 augmented the development and expression of Meth-induced CPP in a developmental model of schizophrenia (Chapter VII). These novel findings contribute to our understanding of the dynamics of mGluR5 system in multiple phases of Meth addiction and reward mediated behaviors, and in the co-morbid stimulant-abusing schizophrenia patient.

Phases of Meth addiction

The addiction phenomenon is a complex and dynamic process. The neurobiology of this process is dictated by many factors, including the nature of the abused drug, the dose and frequency of exposure, the drug experience of the individual, and the involvement of associated behaviors. For purposes of this dissertation project, we adapted a commonly used set of time-related descriptors or phases to categorize our investigations as follows: induction, acquisition/development, maintenance, and expression. The induction phase simply refers to the initial administration of Meth and the behavioral response. The consequences of this initial or acute administration may persist long after Meth is administered. We have assessed two behaviors which occur subsequent to the repeated treatment of Meth, CPP and motor sensitization. During *acquisition,* Meth is repeatedly paired with a context and the rewarding properties of Meth then become associated with these environmental cues. Repeated Meth treatment also results in a progressive enhancement in motor activity and we will refer to the phase between the first and last repeated treatment as the *development* of motor sensitization. Neuronal adaptations that are *induced* and *acquired/developed* are then *maintained* after Meth is no longer administered. Behaviors are then *expressed* under the appropriate conditions where Meth or Meth-related cues are present. Each of these phases provides valuable information as to how neuronal adaptations occur throughout the addiction process.

Induction

The initial Meth administration serves as a stimulus that induces behavioral and neurotransmission responses. The consequences of this stimulus can persist beyond the lifetime of the stimulus itself, and with repeated exposures these consequences can summate. For example, a single treatment with psychostimulants including cocaine, amphetamine, and Meth increases motor function and, with repeated intermittent administration, e.g., once daily, these responses are enhanced or 'sensitized'. We are interested in the mGluR5 neurobiology that is associated with development of sensitization, and so we studied outcomes measured during the induction period and soon after sensitization had been achieved. To study effects during induction, we have assessed changes in Meth-induced behaviors and the mGluR5 system within the inter-dosing interval used in our behavioral paradigms.

When rats are given an acute injection of Meth, motor activity is increased compared to saline treated animals. This hyperactivity was observed in normal rats and was reduced with the mGluR5 NAM, MTEP (Chapter VI). Our laboratory also shows that a single administration of Meth can induce CPP that can persist and be expressed 3 days later (Herrold et al. 2009). Meth therefore, serves as a strong stimulus which *induces* neuronal and behavioral changes that persist after the drug is withdrawn from the system.

Hyperactivity in response to stimulants is a common feature of schizophrenia. Our results agree with this clinical assessment as both repeated,

escalating Amph treated as well as isolation reared rats displayed increased motor activity to an initial Meth injection compared to social reared control animals. The implication of these data in the model of co-morbidity, schizophrenia and Meth addiction will be discussed later.

To fully understand how the mGluR5 system may adapt within the inter-dosing interval used in our behavioral paradigms, we assessed mGluR5 surface expression levels 24hrs after Meth administration. This assessment tells us if basal levels of glutamate receptors are changed as a result of drug injection. We determined that mGluR5 receptors were unchanged 24hr after a single Meth injection, but down-regulation of these receptors with mGluR5 NAM treatment prior to Meth administration reduced the acute hyperactive response to Meth (Chapter VI). Thus, while the function of mGluR5 is necessary for the induction of Meth behavior, changes in protein levels of these receptors are not. Shaffer and colleagues report that mGluR5 protein levels are increased in the membrane fraction of mPFC tissue 1hr after acute Amph (5mg/kg). However, these changes are transient and mGluR5 protein levels are normalized by 5hrs post-Amph treatment (Shaffer et al. 2010). It is therefore possible that mGluR5 levels assessed in the current study would have been initially up-regulated, had tissue been assessed at an earlier time-point. To determine if the acute Meth treatment employed in our study truly replicated the findings of Shaffer and colleagues, rats must be sacrificed and mGluR5 surface expression assessed 5hrs following Meth (1mg/kg) administration.

Acquisition/Development

With repeated treatment, the context associated with Meth becomes significant or salient to the individual. We are interested in the neuronal adaptations that occur during this salience attribution, i.e., how the association between the reward of Meth and the Meth context is *acquired*. Meth-induced motor hyperactivity can also progressively enhance with repeated administration such that the activity induced by the last Meth administration is larger than activity induced by the first, i.e., *development* of motor sensitization. The administration of the mGluR5 NAM, MTEP, prior to repeated Meth administration blunted the development of Meth-induced motor sensitization, a novel finding of the current dissertation project (Chapter VI). The findings from the current studies with CPP and motor sensitization align with the established role of mGluR5 in the acquisition phase of other addiction models. It is known that mGluR5 regulates the development of Meth-induced CPP (Miyatake et al. 2005) and the acquisition of Meth self-administration (Osborne and Olive 2008). Furthermore, mGluR5 knock-out mice fail to develop cocaine-induced motor sensitization. So it is clear that mGluR5 is involved in the development of motor sensitization and this action is not stimulant-specific. We extended this work to ascertain if the role of mGluR5 in Meth-induced behaviors in rat was altered in models of schizophrenia. In the isolation rearing model, where initial deficits in Meth-induced CPP were found, the administration of the mGluR5 PAM, CDPPB, enhanced task acquisition (Chapter VII). Showing that the agonist can promote other forms of learning, CDPPB also facilitates the acquisition of extinction

learning in cocaine CPP treated rats (Gass and Olive 2009). Further discussion of these data in co-morbidity models will follow.

Anatomy and cell biology of induction / acquisition behaviors

The acquisition process of associative learning is thought to involve the transfer of sensory information from the cortex to the NAc, VP and the hippocampus *via* glutamatergic afferents (Christie et al. 1985;Fuller et al. 1987;Sesack et al. 1989). All of these regions also receive dopaminergic input from the VTA and project to motor output regions regulating activity levels. When Meth is administered, extracellular glutamate concentration levels increase in the mPFC and dopamine increases in the NAc (Shoblock et al. 2003). Activation of mGluR5 and dopamine D1 receptors results in an array of transduction cascades which can converge to enhance common secondary and tertiary messengers such as intracellular calcium (Abe et al. 1992;Surmeier et al. 1995) and the phosphorylation of cAMP response element binding protein (CREB) (Dudman et al. 2003;Mao et al. 2007;Roberson et al. 1999;Voulalas et al. 2005). For example, mGluR5 activation results in phosphoinositide hydrolysis and subsequent activation of phospholipase C (PLC) and protein kinase C (PKC) (Abe et al. 1992;Joly et al. 1995), which ultimately can increase intracellular calcium. Activity of PLC and PKC both regulate the development of CPP (Aujla and Beninger 2003;Cervo et al. 1997;Narita et al. 2004). Activation cAMP-dependent protein kinase (PKA) *via* dopamine D1 receptor activation leads to

enhance calcium influx through cAMP-dependent protein kinase (PKA) phosphorylation of ionotropic NMDA receptors (Das et al. 1997). This D1R process plays a role in the acquisition of stimulant-induced associative learning (Beninger and Gerdjikov 2004;Gerdjikov et al. 2007). Activation of both D1 and mGluR5 can increase cAMP-response element binding protein (CREB) (Dudman et al. 2003;Mao et al. 2007;Roberson et al. 1999;Voulalas et al. 2005). CREB phosphorylation can increase protein synthesis and enhance cellular indices of memory, e.g., long term potentiation (LTP) (Bourtchuladze et al. 1994). In the NAc and hippocampus, mGluR5 is necessary for LTP (Bikbaev et al. 2008;Schotanus and Chergui 2008). High frequency stimulation induced LTP in NAc slice preparations is dependent upon mGluR5 and dopamine D1 receptors (Schotanus and Chergui 2008). Induction of LTP in the NAc and dentate gyrus subfield of the hippocampus is inhibited by mGluR5 blockade (Bikbaev et al. 2008;Schotanus and Chergui 2008); both regions are important for the induction of stimulant-induced CPP. Bikbaev and colleagues also revealed that *in vivo* (i.c.v.) treatments of the mGluR5 NAM, MPEP, inhibit acquisition of spatial/working and reference memory in rats, and reduce LTP in *ex vivo* hippocampal slices (Bikbaev et al. 2008). Our data and the literature, concur with the idea that Meth administration indirectly enhances glutamate transmission, and *via* mGluR5 activation in limbic brain regions, promotes the association between the rewarding properties of drugs and the context in which they are administered.

Maintenance

After the acquisition of stimulant-induced associative learning, the stimulant-related memories are maintained for protracted periods. Memory maintenance is a dynamic process. This dissertation project contributed to the understanding the role of mGluR5 in the maintenance of Meth-context associations. We found that mGluR5 was necessary for the long-term (Chapter IV), but not short-term (Chapter III) maintenance of Meth-induced CPP. There are two likely explanations for these findings. First, the mGluR5 system has adapted and perhaps up-regulated over the two week withdrawal from Meth conditioning. Second, the memory of Meth-associations has become more vulnerable to deterioration over time. We included a vehicle control to account for the second factor, and we have biochemical evidence to support that an up-regulation in reward related brain regions occurred 14 days after repeated Meth administration.

Our behavioral and biochemical findings converge upon an important role for mGluR5 in the VP in the *maintenance* of stimulant-mediated behaviors. We found that 14 days after a sensitizing treatment regimen of Meth, the mGluR5 S/I ratio was up-regulated in the VP without a change in surface. This change in the ratio was likely due to a modest decrease within the intracellular pool (Chapter VI). When mGluRs are removed from the membrane surface they are generally either desensitized and rapidly recycled back to the membrane surface, slowly recycled and kept within the endosome of the cell or trafficked to the lysosome and degraded (Dhami and Ferguson 2006). Therefore in the current study,

mGluR5 levels are sustained within the membrane surface of VP cells, while the intracellular mGluR5 proteins are removed or degraded. VP neurons appear to be adapting to the changing glutamate environment to maintain surface levels of mGluR5; yet, surface levels are not scaled up. Interestingly, administration of mGluR5 NAMs blunt Meth-induced CPP at the same withdrawal period that the mGluR5 S/I ratio was up-regulated in the VP. Though levels of the surface mGluR5 component were unaltered, this does not preclude changes in the function of mGluR5 in the VP at the level of downstream signaling mechanisms, for example. Our laboratory has demonstrated that ionotropic glutamate receptors in the VP are necessary for the expression of morphine-induced CPP and motor sensitization (Dallimore et al. 2006), and the work in the current dissertation project suggests that mGluR5 in the VP may be critical for the expression of Meth-induced CPP and motor sensitization as well. In Amph-sensitized rats, VP neurons increase in firing during the presentation of an Amph-associated cue (Tindell et al. 2005). These converging data suggest that modulation of VP neuronal activity by mGluR5 may play an important role in the maintenance and subsequent expression of stimulant-induced associative learning. Exciting future studies with intra-VP injections of an mGluR5 NAM would verify the necessity of mGluR5 during long-term maintenance to express Meth-induced CPP.

Pre-clinical electrophysiological and biochemical experiments (for review, see (Kalivas and Hu 2006)) and human brain imaging studies (Ernst and Chang 2008) suggest that the PFC becomes hypoactive during extended withdrawal

from stimulants. Our data provide one mechanism by which this may occur following repeated Meth administration. We found that 14 days after repeated Meth treatment, surface to intracellular ratio levels of the GluR2 AMPA receptor subunit were increased. This increase corresponded with a decrease in the $STEP_{61}$ protein, while GluR1 AMPA receptor subunits remained unchanged (Chapter VI). We determined that the alterations in GluR2 and $STEP_{61}$ levels were a result of mGluR5 activation during repeated Meth administration (Chapter VI). These findings demonstrate that though mGluR5 surface expression levels were not changed after an initial Meth injection, the activity of these receptors during induction of Meth-induced sensitization likely influences persistent molecular events resulting in alterations in ionotropic glutamate receptor distribution. An increase in the surface expression of GluR2 AMPA receptor subunit without an alteration in the GluR1 subunit would lead to a decrease in calcium permeability and inward rectification of mPFC neurons, known characteristics of GluR2 subunits (Buldakova et al. 1999). Levels of GluR1 and GluR2 mRNA and protein are expressed throughout the layers of the cerebral cortex(Martin et al. 1993;Sato et al. 1993). However, GluR2 levels are generally, modestly higher than GluR1 in the cortex (Sato et al. 1993). Therefore, an increase in the S/I ratio of GluR2 alone would not change overall excitability. GluR2 receptors In conclusion, after 14 days from Meth conditioning, mPFC neurons are likely in a calcium-impermeable state that is regulated by mGluR5.

Expression and biochemical response to Meth-related cues

After Meth-associated memories have been acquired and maintained, they can be expressed under the appropriate conditions, such as the presentation of salient contextual cues previously paired with the rewarding properties of Meth. Activity of mGluR5 necessary for the expression of Amph-induced CPP (Herzig et al. 2005) and cue as well as Meth-induced expression of Meth-seeking behavior (Gass et al. 2009). In the current dissertation project, we also demonstrated that augmenting mGluR5 signaling enhanced expression of Meth-induced CPP in isolation reared rats (Chapter VII, further discussed in following sections). Therefore, mGluR5 protein surface expression levels were assessed 24hr after Meth-induced CPP was expressed. We determined that mGluR5 surface to intracellular ratio was decreased in the mPFC of Meth-conditioned rats. This outcome may reflect internalization of receptors 24hr after CPP expression during short-term withdrawal (Chapter V). Upon exposure to stimulant-associated cues, extracellular glutamate levels in the mPFC are increased (Hotsenpiller et al. 2001;Hotsenpiller and Wolf 2002). Cellular activation also occurs in limbic structures subsequent to drug-associated cues, measured by increases in the immediate early gene *fos* expression (Brown et al. 1992;Rhodes et al. 2005). With prolonged agonist exposure and cellular activation, the mGluR5 system must adapt, and these receptors are phosphorylated and desensitized *via* PKC (Gereau and Heinemann 1998;Lee et al. 2008). Therefore, the internalization of mGluR5 shown after short-term withdrawal and subsequent to drug-related cue re-exposure is likely reflective of cellular homeostasis.

Activation of mGluR5 enhanced Meth addiction behaviors associated with negative symptoms in schizophrenia-like rats

The addiction behaviors assessed in the current dissertation project, i.e., motor sensitization and CPP, were chosen since they may measure positive and negative symptoms of schizophrenia, respectively. We found that activation of mGluR5 through the use of the mGluR5 PAM, CDPPB facilitated CPP but not motor sensitization behaviors (at least at the dose tested). Therefore, mGluR5 may be involved in the negative symptoms of schizophrenia such as anhedonia, cognitive fragmentation and working memory deficits they may lead to addiction vulnerability in schizophrenia patients.

Our isolation rearing co-morbid model revealed that mGluR5 activation can enhance the acquisition and expression of Meth-induced associative learning in schizophrenia-like rodents, which show an initial deficit in this behavior (current study Chapter VII and (Le et al. 2002;Wongwitdecha and Marsden 1995)). A common characteristic of schizophrenia is hypofrontality. The results of the current dissertation support the proposition that mGluR5 is involved in these dynamics that contribute to the ability of schizophrenia-like rodents to acquire stimulant-induced associative learning. The mPFC is a critical brain region for the induction of stimulant-mediated behaviors, and the isolation rearing model used in this project is characterized by decreased levels of mGluR5 in the mPFC (Melendez et al. 2004) as well as reduced mPFC volume (Day-Wilson et al. 2006). There is clinical evidence to support the role of the mPFC in

anhedonia experienced by schizophrenia patients. Recently, it was shown that metabolic activity in the mPFC is negatively correlated with self reports of anhedonia in schizophrenia patients (Harvey et al. 2010;Park et al. 2009). Therefore, by increasing mGluR5 function in the mPFC *via* CDPPB administration, the ability to experience Meth-induced reward may be increased in isolation-reared rodents. There is also pre-clinical data that support the value of enhancing mGluR5 for treating anhedonia; administration of CDPPB increases sucrose consumption in animals that show a deficit in this behavior induced by the NMDA channel blocker, MK-801 (Vardigan et al. 2010). Our findings are congruent with the pre-clinical and clinical literature suggesting that schizophrenia-like individuals have decreased capacity to experience or respond to reward, and we furthered these concepts to demonstrate that mGluR5 activity is important for this phenomenon.

Though isolation reared rats did not express Meth-induced CPP in a drug-free state, the addition of the Meth cue resulted in a positive preference for the Meth-paired context (Chapter VII). These data demonstrate that while schizophrenia-like rats may be less sensitive to the rewarding properties of Meth or perhaps less able to make an association between Meth and the context in which it was administered, they are capable of making the association when in the same physiological state as the conditioning period. Meth increases extracellular glutamate overflow in the mPFC (Shoblock et al. 2003). Cues previously paired with Meth also increase mPFC glutamate and this is enhanced when Meth is given during cue exposure (Qi et al. 2009). Thus, increased mPFC

glutamate may have contributed to the ability of Meth administration during CPP test to promote place preference in the current study.

The CPP procedure involves components of reward as well as associative learning. Another negative symptom, along with anhedonia, central to mPFC abnormalities of schizophrenia which may contribute to the deficit in Meth CPP is working memory deficits (Goldman-Rakic and Selemon 1997). Moreover, working and recognition memory deficits show improvement with by mGluR5 PAMs such as CDPPB (Uslaner et al. 2009). Our data also demonstrate, for the first time, that though schizophrenia-like rodents demonstrate initial deficits in Meth-induced CPP, this behavior was acquired and then was expressed when 'salient signaling' was amplified. That is, when the drug-cue, Meth, is administered prior to the CPP test, all of the signals present during the acquisition phase are present and under these conditions, the isolation reared rats were capable of expressing CPP. CDPPB co-administered with Meth during conditioning, enhances mGluR5 signaling, and this strengthened the acquisition of Meth-induced associative learning so that preference was expressed by isolation reared rats in a drug-free state. Therefore, the glutamate system may contribute to the state-dependency of Meth-induced CPP in isolation reared rats and enhances the neuronal signaling important for associative learning.

Critical discussion of co-mobidity behavioral models

Though repeated, escalating Amph administration and isolation rearing are two very different means of inducing a schizophrenia-like brain state and

sensorimotor gating deficits, we demonstrate that in both pharmacological and developmental models of schizophrenia, sensorimotor gating deficits associated with schizophrenia were negatively related to Meth-induced associative learning. The isolation reared rats and those subjected to repeated, escalating Amph treatments showed PPI deficits. These behavioral responses to Meth are consistent with deficits in sensory processing and cognitive fragmentation in schizophrenia patients. Thus, PPI deficits may serve as a predictor for stimulant-mediated dysregulation. And so doing, PPI deficits may provide a means of screening for potential vulnerability to substance use disorders as well as schizophrenia itself. We also found that Amph- and isolation rearing-induced deficits in PPI are related with an enhancement in Meth-induced hyperactivity. Our findings are supported by the literature that indicates schizophrenia-like rodents show an enhanced response to stimulant-induced motor sensitization (Berg and Chambers 2008;Chambers and Taylor 2004) but a deficit in stimulant-induced associative learning (Le et al. 2002;Wongwitdecha and Marsden 1995). Another developmental rodent models of schizophrenia, which implements neonatal hippocampal lesions (NVHL) is also characterized by an increase in drug-taking behavior measured in self-administration paradigms (Brady et al. 2008;Chambers and Self 2002). Though not directly correlated with stimulant seeking or taking behavior, NVHL rats show PPI deficits (Lipska et al. 1995). These data call into question what aspects of addiction are modeled by CPP, motor sensitization, and self administration and how these addictive behaviors manifest and contribute to substance use disorders in the co-morbid

schizophrenia patient. The addiction model of CPP utilizes reward and associative learning mechanisms, both of which relate to negative symptoms of schizophrenia. Therefore, it is logical that our data demonstrate a negative correlation between sensorimotor gating deficits and Meth-induced place preference. The self-administration completed in the laboratories of Chambers and O'Donnell stress that schizophrenia-like rodents are motivated to perform an operant task for a stimulant reinforcer (Brady et al. 2008;Chambers and Self 2002). The use of a progressive ratio schedule for Meth self-administration nicely demonstrates that schizophrenia-like rats are even motivated to work harder than control animals to receive Meth reinforcement (Brady et al. 2008). The acute effects of Amph (e.g., euphoria, hyper-vigilance) appear to "sensitize" with repeated exposure and can result in psychotic state that is akin to the positive symptoms of schizophrenia (Angrist et al. 1980;Strakowski et al. 1996). Thus, it is also logical that with both models of schizophrenia used in the current project, the motor responses to Meth were greater than (i.e., sensitized) that observed in controls (i.e., social). Therefore, we have determined that deficits in sensorimotor gating induced by two very different means (i.e., repeated, escalating Amph and isolation rearing), produce a phenotype that negatively correlate to the rewarding and positively correlate to the motor effects of Meth.

There are many hypotheses as to why schizophrenia patients take illicit substances. One of which is that schizophrenia patients are "self medicating", perhaps due to a reduced capacity to feel reward, or anhedonia (Khantzian 1997). Another hypothesis is that schizophrenia patients are more vulnerable to the

rewarding effects of abused substances (Krystal et al. 1999;Tseng et al. 2009). These two hypotheses may go hand-in-hand: that is, negative symptoms may predict as well as play a causal role in stimulant use. Both working memory and anhedonia occur and predict substance abuse likelihood in schizophrenia patients (Potvin et al. 2008a;Potvin et al. 2008b). Therefore, it may be hypothesized that schizophrenia patients must take more stimulants in order to experience their rewarding or reinforcing effects. Our studies also demonstrate that the mGluR5 system may be critical in fine tuning the signaling necessary to amplify the effects of stimulants in schizophrenia-like rats. Therefore, modulation of mGluR5 may be a potential therapeutic target not only for Meth addiction but also Meth addiction in the co-morbid schizophrenia patient.

In conclusion, the current dissertation project makes an important contribution to the current understanding of mGluR5 function and expression in the maintenance of Meth behaviors. Furthermore, these data and their interpretation elucidate a role of mGluR5 in modulating rewarding signaling in Meth abuse, schizophrenia co-morbidity.

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VITA

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In 2007, Amy received a pre-doctoral Ruth L. Kirchstein National Research Service Award from the National Institute of Health / National Institute of Drug Abuse (NIDA) to support her dissertation research project. Amy has been a part of many research collaborations during her time in the Napier lab and the Center for Compulsive Behavior and Addiction at Rush University which is reflected by her diverse publication/presentation history. Amy received competitive travel awards to attend and present her research at international conferences from the Committee on Women in Neuroscience of the Society for Neuroscience (2007 Society for Neuroscience conference, San Diego, CA) and NIDA (2009 College of Problems on Drug Dependence, Reno, NV).

After completing her dissertation research, Amy will be pursuing a career in translational neuroscience in academia.